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THE UNIVERSITY OF ALBERTA

ANTIGEN RECOGNITION BY POLY 18 SPECIFIC T CELL HYBRIDOMAS

by

MICHEL BOYER

A thesis submitted to the faculty of graduate studies and research in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In

MEDICAL SCIENCES (IMMUNOLOGY)

DEPARTMENT OF IMMUNOLOGY

EDMONTON, ALBERTA

SPRING 1989

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of which I am a co-author.

Best wishes,

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Antigen recognition by poly 18-specific T cell hybridomas

submitted by Michel Boyer
in partial fulfilment of the requirements for the degree of
Doctor of Philosophy
in Medical Sciences (Immunology)

Supervisor

Date: December 5, 1988

DEDICATION

To my father GASTON R. BOYER whose encouragement has inspired me throughout the years.

ABSTRACT

Most helper T cells recognize antigen in the context of Class II Major Histocompatibility Complex encoded products (Ia) on the surface of antigen presenting cells (APC). Recognition generally follows an antigen processing step performed by the APC, and is thought to involve the formation of a trimolecular complex between antigen, Ia and the T cell receptor. Ordered secondary structure is believed to be an important feature of the antigen, in this complex.

The antigen recognition requirements of T cell hybridomas derived from BALB/cCr immune responses to the synthetic polypeptide antigen poly 18 [poly-EYK(EYA)$_5$] were examined. Two I-A^d restricted clonotypes were identified from the poly 18-specific T cell population. The first, exemplified by clone A.1.1, is specific for the lysine-containing portion of poly 18; the second, represented by clone B.1.1, recognizes preferentially, but not exclusively, a determinant without lysine. Using synthetic peptides, the minimum-size sequence capable of eliciting a response from the T hybrids was found to be 15 amino acids long. Poly 18 and stimulatory peptide analogs were recognized without prior processing by the antigen presenting cell. Critical amino acid residues of the minimum-size peptide EYK(EYA)$_4$, which stimulates A.1.1 and B.1.1, were identified by measuring T hybrid responses to peptide analogs with single amino acid

substitutions. Of the 6 critical residues found, four were required for the stimulation of both A.1.1 and B.1.1. They are thought to interact with Ia since they were necessary for both hybridomas and because peptides substituted at these positions failed to competetively inhibit the activation of the T hybrids by EYK(EYA)$_4$. The other two critical residues were unique to each hybridoma and were implicated in interactions with both the T cell receptor and Ia. One of these was found to interact with a neighbouring amino acid within the antigen, in a way that critically affected the response of A.1.1. This identified a novel role for a critical residue, distinct from that of interacting with Ia or the T cell receptor. Circular dichroism measurements revealed little ordered secondary structure in the peptide antigens used in these studies.

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
BSA	Bovine serum albumin
CD	Circular dichroism
CFA	Complete Freund's adjuvant
ChlQ	Chloroquine
CPM	Counts per minute
DMF	N,N'-dimethylformamide
DNP	Dinitrophenyl
G.A.	Glutaraldehyde
HPLC	High performance liquid chromatography
IL-2	Interleukin 2
KLH	Keyhole limpet hemocyanin
MHC	Major Histocompatibility Complex
ND	Not determined
PPD	Purified protein derivative
S.D.	Standard deviation
TCR	T cell receptor
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
TLC	Thin layer chromatography

CHAPTER 1

Introduction

One of the hallmarks of the immune system is its ability to single out pathogens for destruction. This specificity of the immune response results from a property invested in T and B lymphocytes, which enables them to distinguish between self and foreign, to recognize invaders and to direct an attack toward them. Recognition of antigen by lymphocytes has been studied extensively, but much more progress has been made in understanding how it operates in B cells than in T cells. This is in part because, in the latter, recognition is the end point of a complex process involving more than one cell type.

ANTIGEN RECOGNITION BY T CELLS

Most T cells recognize a processed form of antigen. Evidence of this was first obtained when Gell and Benacerraf [1] highlighted an important difference between delayed type hypersensitivity and secondary humoral responses. They observed that the latter could not be elicited to a native antigen unless the antigen was administered in the same form upon challenge as when the animal was immunized. In contrast, delayed type hypersensitivity responses were readily obtained

by injecting either native or denatured antigen at the time of challenge. Thus, in the case of cell-mediated immunity, later found to be due to T cells, antigen is being recognized although its tertiary structure is disrupted. Many reports followed in which it was shown that T cells respond to denatured or fragmented antigens [2-7] as well as to the native antigen used for immunization. Therefore, T cells appear to prefer "sequential determinants" [8] over "conformational epitopes".

Role of the antigen presenting cell: determinant selection

Experiments to analyse the T lymphocyte response to antigen, in the guinea pig, revealed the requirement for an "antigen-binding cell" whose function was to present the antigen to T cells [9,10]. Furthermore, these studies showed that the antigen-binding cell had to be histocompatible with the T cell and of the responder phenotype [11,12]. Similar observations were also made in the mouse [13-15]. Studies by Rosenthal's group on the immune response to insulin in guinea pigs showed that strain 2 and 13 macrophages presented different segments of the antigen to the T cells. The authors speculated that one of the functions of the antigen-presenting cell was to handle or process the antigen to display it in an immunogenic form for the T cell [16,17]. This constituted an essential part of the determinant selection hypothesis which stated that immune response (Ir)

gene effects could be attributed, at least in part, to the antigen-presenting cell's ability to select certain determinants of an antigen and to present only these to the T cell. Ir gene products were thought to be receptors with broad specifity "which recognize molecular shape and thus have the unique ability to focus or orient distinct regions of the antigen for presentation to the T cell [16]".

Antigen processing

Handling of antigen by macrophages was studied extensively by Unanue [18]. Early studies using ^{125}I-labelled hemocyanin showed that most of the protein was taken up and catabolized while about 20% was released or remained associated with the cell, either on its surface or in an internal pool. There was no correlation between the amount of antigen catabolized and the immunogenicity of the macrophage associated protein. Somehow, the antigen remained available on the macrophage for some time to stimulate an immune response. Similar observations were made in the guinea pig system using albumin as the antigen [19]. In a series of experiments to determine what relevance antigen catabolism might have for T cell stimulation, Ziegler and Unanue used a short term assay in which they measured the binding of *Listeria*-immune T cells to antigen pulsed macrophages [20]. They found that the T cell's ability to bind to the

presenting cell lagged behind the uptake of antigen by 30 to 60 minutes. This time lag was necessary to allow processing of the antigen by the macrophage, a temperature-sensitive and energy requiring step, which could be blocked by fixation of the cell with paraformaldehyde. These experiments suggested that antigen catabolism was an important requirement for antigen-specific T cell binding to APC, a conclusion that was confirmed in a subsequent study using ammonia and chloroquine [21], agents which inhibit intracellular degradation of antigen by the macrophage. Studies by Chesnut et al. showed that ovalbumin-specific T cells which responded to denatured OVA were the same cells that recognized native OVA, suggesting that the native protein was likely being altered and seen as denatured [22].

Nature of the processing event

By fixing the antigen-presenting cell with glutaraldehyde to prevent processing and adding chemically or enzymatically cleaved antigen, Shimonkevitz et al.[23,24] were able to stimulate a number of ovalbumin-specific T cell hybridomas which did not respond to native or denatured OVA on fixed APC, thereby showing that antigen fragmentation was a necessary and sufficient step for T cell activation, at least in this case. Studying the response to hen egg-white lysozyme (HEL) by two T cell hybridomas, using

paraformaldehyde or chloroquine treated macrophages, Allen and Unanue [25] found that fragmentation of the antigen was necessary for one hybridoma but that denaturation was sufficient to elicit a response from the other. Streicher et al. [26] also found that unfolding of the antigen was sufficient to obtain a response from a T cell clone specific for myoglobin, leading them to suggest that the role of processing was to expose immunogenic sites normally buried in the native protein. This idea is echoed in a study of the processing requirements of a cytochrome c-specific T cell hybridoma where it is suggested that effective antigen/T cell interaction necessitates disruption by the APC of an internal bond between two residues, Glu 61 and Lys 99, of the cytochrome c molecule [27]. Additional insight into the functional importance of processing came from the response of T cell clones to hen and pheasant lysozyme, two closely related molecules which share the same antigenic determinant but elicit very different responses due to substitutions distant from the epitope [28]. These results were interpreted to mean that there was differential processing of the antigens controlled by the distant substitutions and resulting in different T cell reactivities.

The experimental protocols which blocked the processing ability of the antigen presenting cells opened the way to the unequivocal identification of minimum size antigenic determinants recognized by T cells. A further refinement was contributed by the development of synthetic membrane systems

immobilized on a solid support [21,29,30]. Using a planar membrane containing Ia alone, Watts et al. confirmed that only fragmented ovalbumin could stimulate the OVA-specific hybridoma 3DO-54.8.

The B cell as APC

For many years, it was believed that only phagocytic cells could process antigen. While this may still be true of large particulate antigens, such as bacteria or viruses, several laboratories have shown that B cells and other non-phagocytic cells can also process soluble antigen. Chesnut and Grey published evidence that B cells capable of binding antigen through their Ig receptor could present it to T cells in an MHC-restricted manner [31]. McKean et al. [32] first demonstrated that a pure B cell tumor population was efficient at presenting antigen to T cells. Several studies followed, confirming and extending these observations, both in vitro [33-39] and in vivo [40]. The presenting efficiency of B cells was found to be greatly increased if they were antigen-specific, suggesting that surface Ig bound and focussed the antigen for optimal T cell interaction [41]. In addition, it appears that B cells must be activated to present antigen [35], although resting B cells have also been shown to have this capacity, but in a more limited radiosensitive manner [42].

Antigen processing *in vivo*

An important question to be addressed about processing is whether it plays a significant role in vivo. Studies on the immune response to hen egg lysozyme in different mouse strains have shown that processing events can orient the response to a selected number of the available determinants in a protein molecule, and in some cases can dictate whether T helper or T suppressor cells will be induced [43]. Fox et al. [44] described a processing event important for the in vitro T cell response to a substituted cytochrome c peptide which appeared to be operating in vivo as well. The potency of the antigen in vitro was dependent on processing and its immunogenicity in vivo suggested that it might be processed there as well. More convincing evidence was provided by an elegant experiment in which it was found that a T cell hybridoma specific for a processed form of the *Hbb*^{d} allele of hemoglobin could be stimulated by APC freshly removed from an *Hbb*^{d}-bearing animal without any added antigen, demonstrating that the APC carried processed self hemoglobin on their surface [45]. Control experiments, involving paraformaldehyde-fixation of the antigen presenting cells immediately upon excision from the animal, showed that the processing event was occurring in vivo before the APC were put in culture.

REQUIREMENT FOR CORECOGNITION OF ANTIGEN AND MHC

Antigen recognition by T lymphocytes occurs at the surface of accessory cells in the context of MHC encoded gene products. This requirement for histocompatibility, called restriction after the landmark experiments of Zinkernagel and Doherty [46], sparked a vigorous debate about the molecular structures by which the T cell could recognize both antigen and the polymorphic MHC. Three basic models were proposed: in the first, T cells were thought to express two receptors: one specific for antigen and the other for the MHC product on the presenting cell [47-49]. In the second model, a single receptor with two combining sites would recognize antigen and MHC independently [50-52]. Finally in the third, one receptor would be made by the T cell which would be specific either for the MHC altered by antigen [53] or for a complex of antigen and MHC [54,55].

Lack of independent recognition of antigen and MHC

In an experiment to address this question, Kappler et al. fused an ovalbumin-specific, H-2^a-restricted T cell hybridoma with KLH-specific T lymphoblasts restricted to H-2^f [56]. They reasoned that if T cells see antigen and MHC with separate receptors, the hybridoma they had produced should be capable of responding to OVA in the context of H-2^f and to KLH in the context of H-2^a, as well as having the original

specificities of the fusion partners. The new specificities were not found, suggesting that antigen and MHC were being recognized by one receptor.

Experiments by Hünig and Bevan [54] were in agreement with this interpretation and suggested that antigen recognition by cytotoxic T cells was not radically different from that by helper T cells. A CTL clone specific for H-2^k plus X (a DBA minor histocompatibility antigen) was found to cross-react with H-2^d plus Y (a Balb minor antigen). Since it would not respond to H-2^k plus Y or H-2^d plus X, it was concluded that antigen and MHC were not being seen independently of each other by the T cells, and were probably recognized as a complex by a single receptor.

INTERACTIONS BETWEEN ANTIGEN AND Ia

Evidence to support the one-receptor model was also provided by studies which strongly suggested that antigen and Ia interact with each other. Using cytochrome c as a model antigen, for which there are a number of species variants useful for fine specificity studies, Schwartz's group [57] found that the immune response to pigeon cytochrome c in B10.A ($E\beta^k$:$E\alpha^k$) mice generated T cell clones capable of a heteroclitic response to moth cytochrome c. They also found that these clones, and hybridomas derived from them, could respond to moth but not to pigeon cytochrome c in the context of B10.A(5R) ($E\beta^b$:$E\alpha^k$) APC. So the T cells were able to "see"

both pigeon cytochrome c and ($E\beta^b$:$E\alpha^k$) when certain conditions were met but they could not recognize them together. This suggested that antigen and Ia were required to interact with each other for T cell recognition, a requirement which could not be met by pigeon cytochrome c and ($E\beta^b$:$E\alpha^k$). Further experiments showed that some B10.A T cells heteroclitic for moth cytochrome c on syngeneic APC could respond better to pigeon than to moth cytochrome on B10.S(9R) APC, again indicating that the Ia molecule was affecting recognition of the antigen. These findings led to the formulation of a model of T cell recognition in which the T cell receptor, antigen and Ia interact in a trimolecular complex [58].

Competition between antigens for the presenting cell

Competition experiments in which an antigen-specific response can be inhibited by the addition in culture of a non-stimulatory antigen are consistent with the notion that antigen and Ia interact. Guinea pig responsiveness to dinitrophenyl-poly-L-lysine (DNP-PLL) and to a random copolymer of L-glutamic acid and L-lysine (GL) is under Ir gene control. Lymphocytes immune to DNP-PLL could be prevented from responding to the antigen if the presenting cells were preincubated with GL, suggesting that these molecules compete with each other for the Ir gene product on the APC. The interaction between these antigens and the APC showed some specificity as various other synthetic

polypeptides and protein antigens competed less effectively or not at all [59]. Similar competition experiments were performed in the murine system [60]. They showed that the response of T cell hybridomas specific for the terpolymer GAT (L-glutamic acid^{60}-L-alanine^{40}-L-tyrosine^{10}) and restricted to I-A^{d} could be competitively inhibited by the presence of the copolymer GT (L-glutamic acid^{50}-L-tyrosine^{50})in the culture. The evidence indicated that the antigens competed for Ia on the presenting cell, and that this competition was specific since GT inhibited only the GAT response and only of I-A^{d} restricted T cells.

Binding of antigen to Ia in equilibrium dialysis

Direct evidence that antigen and Ia interact was provided by the demonstration that an immunogenic peptide could bind to isolated Ia [61]. The 16 amino acid peptide of lysozyme HEL(46-61) is immunogenic in H-2^{k} but not in H-2^{d} mice. It was labelled and used in an equilibrium dialysis assay with detergent solubilized Ia and was found to associate specifically with I-A^{k} but not with I-A^{d}. The binding was saturable with a dissociation constant, K_D, of approximately 2 μM. Furthermore, unlabelled HEL(46-61) could compete out the test peptide, whereas a non-immunogenic analog was unable to do so. Binding efficiency, however, was found to be low as only 5-6% of peptide bound to I-A^{k}.

These results were confirmed and extended to the ovalbumin system, where it was demonstrated that the immunogenic peptide OVA(323-339) bound specifically to I-A^d, and not to irrelevant Ia molecules [62]. Studies on the kinetics of association and dissociation of antigen and Ia showed that the complexes formed very slowly (k_a~ 1 M^{-1} s^{-1}) but were quite stable (k_d~ $3x10^{-6}$ s^{-1}). When introduced into a planar membrane, these complexes very efficiently stimulated an OVA-specific T cell hybridoma compared to the stimulation obtained when free peptide was given in culture with uncomplexed I-A^d in a planar membrane [63]. These findings emphasized the importance of antigen-Ia interaction for T cell stimulation.

Other binding studies

Similar interactions were demonstrated with other approaches. Using photoaffinity cross-linking, close associations were found between insulin and cell surface Ia [64]. Energy transfer experiments between fluorescein-labelled peptide OVA(323-339) and Texas-red labelled I-A^d indicated that these molecules were within 40 Å of each other in the presence of the specific T cell hybridoma, which suggested that the T cell receptor may stabilize the peptide-Ia interaction [65].

The antigen binding site on MHC

The competition experiments reported by Werdelin [59] and Rock and Benacerraf [60] showed that only certain antigens could compete with each other while others did not, although they were known to interact with the same restriction element. These observations suggested that there might be several binding sites for antigen on Ia. Studying two GAT-reactive, $A_\alpha^b A_\beta^k$-restricted T cell clones, Beck et al. found that only one of these crossreacted to GAT presented by APC of the I-A^b mutant bm12. This was interpreted to mean that more than one restriction site was present on Ia [66,67]. As well, using anti-Ia monoclonal antibodies to block T cell responses, two groups have shown complex patterns of inhibition, in the mouse and guinea pig, suggesting that several interaction sites were available to T cells on Ia [68,69]. Finally, putting together data from experiments involving 10 I-A^k-restricted, HEL-specific T cell hybridomas, a panel of APC with mutated I-A_α^k and I-A_β^k chains, and fragments of HEL or related synthetic peptides, Allen et al.[70] concluded that several distinct domains were recognized by T cells on the I-A^k molecule.

This interpretation, however, was challenged recently and an alternative explanation was proposed. Based on the different reactivities of two I-A^d-restricted T cell hybridomas specific for a common immunodominant region of sperm whale myoglobin, Cease et al. suggested that the two T

cells viewed the same antigen-Ia complex in different ways. This meant that there was no need to postulate more than one antigen-binding site on Ia [71]. These data, as well as those previously reported, did not allow to distinguish between the two hypotheses. However, the single-site model received additional support from the competition studies published by Guillet et al., who found that the response of bacteriophage λ cI protein-specific, I-A^d-restricted T cell hybridomas could be inhibited, not only by analogs of the antigen but also by unrelated peptides which were known to be I-A^d-restricted, such as *S. aureus* nuclease and ovalbumin [72]. Extensive studies correlating competition between peptides for binding to soluble Ia with inhibition of T cell responses by non-stimulatory peptides suggested that a large number of amino acid sequences could interact with a single site on a given Ia molecule [73-76].

Structure of the MHC molecule

Solution of the crystal structure of the human Class I histocompatibility molecule HLA-A2 provided new insights into our understanding of antigen-MHC interactions. The membrane-distal domains α_1 and α_2 of the Class I molecule were found to fold into a platform of 8 antiparallel β-strands topped by 2 α-helices. The latter run parallel to each other and form the walls of a cleft which was found to contain a separate, unidentified structure believed to be a processed peptide

antigen. The size of the cleft could accommodate peptides of 8 to 20 amino acids [77,78]. There appeared to be no other "binding site" on the molecule, but the presence of multiple subsites within the groove cannot be ruled out at this point. Using this structure as a basis, Brown et al. proposed a model for the antigen-binding site of Class II histocompatibility molecules, which is consistent with many serological, functional and molecular studies of Class II antigens [79].

NATURE OF THE T CELL RECEPTOR

The T cell receptor heterodimer

Identification of the receptor eluded investigators for many years, but a major step forward was taken when monoclonal antibodies were generated to clonally expressed structures on the surface of T cells [80-94]. Since they affected T cell responses to antigen, these antibodies were believed, correctly, to be directed at the antigen-specific receptor. One monoclonal antibody, raised against an OVA-specific, I-A^d-restricted hybridoma, predicted the antigen/MHC specificity of an independently derived hybridoma against which it reacted, strongly suggesting that it was directed against the T cell antigen receptor [94]. Furthermore, these antibodies precipitated similar molecules:

80 to 90 kDa glycoproteins, consisting of an acidic 39-46 kDa α-chain disulfide-linked to a more basic 40-44 kDa β-chain, both with constant and variable regions. Each chain also contained intra-chain disulfide bonds [95].

Molecular biology of the T cell receptor

A major breakthrough came with the isolation of the genes encoding the receptor. Using subtractive hybridization, Hedrick et al. isolated cDNA clones for T cell specific membrane proteins which they thought would be the T cell receptor based on four criteria: 1) the genes they had identified were expressed in T cells but not in B cells; 2) the mRNAs for the receptor were found on membrane-bound polysomes; 3) the genes were rearranged in T cells; and 4) they encoded proteins with constant and variable regions [96]. At the same time, a similar gene was identified in a human T cell [97]. Both were identified as encoding the β-chain of the T cell receptor by comparison of their predicted amino acid sequence with the actual sequence of the β-subunit of the REX cell line receptor [98]. Soon after, genes for the α-chain were discovered [99,100] as well as for a third receptor chain, designated γ, whose existence was unsuspected until then [101]. Finally, a fourth gene, called δ, was found to encode a polypeptide chain associated with γ [102-104]. The γ:δ receptor is complexed on the cell membrane with the CD3

molecules and is expressed on thymocytes, on most dendritic T cells in the epithelium and on a small number of peripheral T cells [103-107]. However, the function of γ:δ bearing cells is not known and the nature of the receptor's ligand has not been elucidated.

The genomic organization of the T cell receptor shows many similarities with that of immunoglobulins. The V,J or V,D,J, and C regions which make up a TCR chain are assembled together out of a pool of gene segments which occupy a large expanse of chromosomal DNA. Rearrangements follow the 12-23 rule seen in antibody formation [108], and are performed by looping-out and deletion [109,110], as well as inversion [111]. The β chain locus contains 20 to 30 V region genes. These can rearrange with a previously formed DJ segment, which itself arises from combining one of two D genes to one of 12 J's [112]. There are 2 C-region genes at this locus. The α chain V genes number an estimated 75 to 100 segments which can combine with some 50 J segments and one C. An unusual feature of this locus is that it contains the coding sequences of the δ chain between the V_α and J_α segments [113,114]. These sequences are deleted in α:β-bearing T cells as a result of V_α-J_α joining. There are about 10 V segments capable of combining with 2 D's, 2 J's and one C in the δ locus. The γ chain genes include 3 functional C's each with its own J segment and only seven V's [115,116].

Generation of diversity

In spite of the relatively small number of V genes in the murine T cell receptor pool, compared to the immunoglobulin's 200 V_K and 200-1000 V_H sequences [117], a great deal of diversity can be generated, particularly in the third CDR where V,D and J (or V and J) meet. This is achieved notably by the random addition of nucleotides between the germline encoded segments, in what is called N-region diversification [118], by the large number of J sequences, by imprecision in the joining of segments which results in deletions of nucleotides, and by the possibility of using D regions in all three reading frames [116]. Based on these findings, attempts have been made to estimate the size of the available repertoire of T cell receptors, with widely diverging results: from 10^7 [119] to more than 10^{15} [114] different receptors are thought to be possible.

Structure of the T cell receptor

Identification of the T cell receptor genes in many antigen-specific lines made it possible to derive a great deal of information about the structure of the surface-bound polypeptides. These molecules show many similarities with immunoglobulins, being made up of variable (V), joining (J) and constant (C) regions and, in the case of the β and δ chains of one or more [113] diversity (D) regions.

Hypervariable regions are found in the TCR which, like in immunoglobulins, are encoded by the V genes or are formed by the VJ or VDJ junctions [116,120,121]. These regions are likely the T cell receptor equivalents of the immunoglobulin complementarity-determining regions which form the antigen combining site [122,123], and are presumably engaged in contacting the T cell receptor ligand. The constant region includes a large immunoglobulin-like domain of 90-100 amino acids with an internal disulfide bond which probably stabilizes the structure in a typical immunoglobulin fold [116]. Three additional segments are located at the C-terminal side of the constant region: a short hinge region containing a cysteine for interchain bridging, a transmembrane region which has a positively-charged lysine thought to interact with the CD3 polypeptides and a cytoplasmic tail.

Concomitant transfer of TCR genes and antigen specificity

With its many similarities to immunoglobulin, and its exclusive expression in T cells, there seemed little doubt that the molecule identified by monoclonal antibodies and molecular biology was the T cell receptor for antigen. The experiments that finally confirmed it consisted in transferring the α and β chain genes into a T cell and demonstrating that the antigen specificity associated with

the transferred genes was newly acquired by the recipient T cell. This was first performed in a CTL hybridoma, which was specific for the hapten SP [3-(*p*-sulphophenyldiazo)-4-hydroxyphenylacetic acid] and K^k. The new α and β chain genes came from a fluorescein-specific, D^d restricted CTL clone. The transfected cell, BD7-S17, transcribed the introduced genes, retained specificity for SP on K^k, and acquired that for fluorescein on D^d [124]. As Kappler et al. had found earlier [56], no mixed specificity was observed: that is BD7-S17 did not lyse targets bearing SP and K^k or Flu and D^d. Therefore, recognition of both antigen and MHC was being performed by a single heterodimer. Its specificity was determined by the contribution of a unique α chain paired to a unique β chain.

Similar results were obtained when the receptor of a Class II restricted T cell was transferred to another cell. In this case, the α and β chain donor was the pigeon cytochrome c, I-E^k restricted mouse T cell hybridoma 2B4, and the recipient was either the T3-positive human T cell tumour Jurkatt or a β chain-negative variant of it. Introducing the murine α and β chains, alone or together, in the two Jurkatt lines showed that human-murine heterodimers could be expressed on the cell surface as well as the murine heterodimer. However, only the mouse α-β chains transferred 2B4 specificity to Jurkatt cells, showing that neither chain alone was responsible for antigen or MHC recognition [125].

Composition of the combining site for antigen and MHC

An interesting question raised by the ternary interaction model is whether the α- and β-chains of the T cell receptor have distinct functions in recognition of the antigen and MHC. That is, does one of the chains interact with MHC while the other sees antigen or do both chains bind both ligands? Comparing the primary sequences of the antigen receptors of a number of cytochrome c-specific clones, Fink et al. found a correlation between V_β-J_β usage and specificity for a particular I-E molecule [126]. Kappler et al. also suggested that the β chain may be primarily involved in Ia interaction, based on the observation that V_β17a imparts reactivity to I-E in most T cells using it [127]. On the other hand, the same V_β2B4 gene is used by I-E restricted and $I\text{-}A^k$ restricted T cells [128]. In their analysis of TCR gene usage in the cytochrome c response from four mouse strains, Winoto et al. found no simple correlation of MHC restriction with gene segment use. However, V_α11.1 was predominantly used in cytochrome c responsive T cells [129]. In further analysis of cytochrome c-specific clones, a highly selected amino acid sequence including an Asp or an Asn at position 100 was found in the V-D-J region junction, corresponding to the third CDR of immunoglobulin. This sequence was suggested to be critical for T cell specificity and position 100 was implicated in antigen recognition [130].

In an attempt to rationalize the conflicting data, Davis and Bjorkman proposed an alternative model of recognition, in which both chains of the T cell receptor are engaged in contacting MHC and antigen. Three hypervariable regions are postulated to form three immunoglobulin-like complementarity determining regions on each TCR chain. CDR's 1 and 2 are encoded by the V regions and interact with the α-helices of the MHC molecule which border the antigen binding site. The 2 CDR-3 loops are formed by the V-(D)-J region junction and are concerned with contacting the antigen siting in the MHC groove [116].

THE ANTIGEN IN T CELL RECOGNITION

Antigenic determinants recognized by T cells on protein molecules were identified by comparing responses to homologous proteins with known amino acid differences, or by measuring the stimulatory ability of fragments of the antigens or of synthetic peptide analogs. An appreciable number of determinants were characterized that are stimulatory for T cells in guinea pig, human, rat and murine responses. They were found notably in insulin [17,131], cytochrome c [6,132], hen egg lysozyme [133-135], myoglobin [136-138], influenza nucleoprotein [139] and hemagglutinin [140], bacteriophage λ cI protein [72], *Staphilococcus* nuclease [5], ovalbumin [24], and others. In many cases, only a limited number of antigenic sequences were identified in a

given protein [17,70,133-135,141]. This led to the concept of immunodominant sites, which focus the T cell response at the expense of other possible epitopes. This bias imposed on responses could be ascribed to a number of causes, such as regulation, processing, T cell repertoire, or the ability of peptides to interact with MHC products [43].

The response of T cells to antigen was found to be exquisitely specific in some cases but could appear wildly degenerate in others. A peptide differing from the immunogen by a single amino acid could fail to stimulate a response [6]. On the other hand, a hybridoma specific for a 13 residue bacteriophage λ cI peptide was able to respond to a substituted ovalbumin peptide with 9 amino acid differences [72].

Epitopes and agretopes

Analysis of the T cell response to cytochrome c allowed the identification of distinct subregions on a C-terminal peptide containing the antigenic determinant. Using substituted synthetic peptides, Hansburg et al. found that replacing Lys 99 by a Gln changed the memory of the responding T cell population, suggesting that this position was critical for interaction with the T cell receptor [142]. On the other hand, position 103 controlled the response that could be obtained with a number of degenerate clones and hybridomas on B10.A and B10.A(5R) antigen presenting cells.

This suggested that position 103 was involved in interaction with Ia [142,143]. These subsites of the antigen were called epitope and agretope, respectively [58].

Role of secondary structure in antigenicity

An interesting feature of the two functional regions is their close proximity to each other. Similar observations were made in mapping the T cell receptor and Ia interaction residues of HEL(52-61) [144] and OVA(323-336) [145]. In the first case, the sites were composed of distinct but interspersed amino acid residues which were postulated to adopt an α-helical conformation segregating Ia from T cell receptor interaction residues. For the OVA peptide, three residues appeared to belong to both functional regions. It was suggested that they interacted simultaneously with the T cell receptor and Ia and that, as a result, the structure of the peptide in the binding site was extended or approached a β-sheet conformation.

The suggestion that secondary structure may play a role in T cell recognition was put forward to explain why no T cell response could be obtained to the moth cytochrome c peptide 97-103 although it contained the critical interaction positions 99 and 103. The full T cell response to moth cytochrome c required the sequence of residues 88-104, and it was postulated that residues 88-98 served to induce an α-

helical conformation in the peptide resulting in appropriate orientation of the critical residues [146].

Analysing a number of known antigenic sites for their structural potential, DeLisi et al. found that most of them displayed amphipathic periodicity in their primary sequence, which was consistent with an α-helical conformation. They proposed that this was a general property of T cell antigenic sites which could be used to predict which amino acid sequences of a protein antigen would elicit T cell responses, a potentially useful tool to develop vaccines [147].

Common structural patterns in antigens

In the same vein, the accumulating evidence of direct antigen binding by Ia prompted an examination of T cell antigenic determinants with a view to detecting common structural features that could account for the broad specificity of the MHC molecule. Rothbard examined 30 published T cell epitopes and in 29 of them found one of two similar motifs. These are 4 or 5 amino acid sequences composed of a charged residue or glycine followed by 2 hydrophobic residues, then a charged or polar residue in the short motif or a non-polar amino acid followed by a charged or polar residue in the long motif [148]. Using a different approach, Sette et al. [145] also found that peptides with good I-A^d binding capacity shared a common sequence pattern, which overlapped the motif proposed by Rothbard.

A more sophisticated paradigm was later suggested to account for the specificity differences between MHC haplotypes [149]. The proposed motif was again made up of 4 amino acids, with two central hydrophobic residues and, on either side of these but two positions removed, an amino acid generally conserved within a given haplotype. If the antigen was modeled to adopt an α-helical conformation, these four residues would fall on the same face of the helix, where they could interact with the restriction element. This model predicts that the motifs of two antigens with the same Ia restriction should be interchangeable. In an experiment to test this, Rothbard et al. constructed two hybrid molecules: one of the influenza matrix peptide 17-29 with the motif from influenza hemagglutinin 307-319, and the reciprocal hemagglutinin with matrix motif. Both parent molecules are DR-1 restricted and stimulate non cross-reactive T cell clones. Results showed that the matrix specific clone could be stimulated by the hybrid antigen containing the hemagglutinin motif and vice-versa [150]. However, several amino acids in addition to the 4 residue motif had to be transferred from one antigen to the other in order to elicit a response, suggesting that other factors were at work.

Investigating the interaction of a variety of peptide antigens with I-A and I-E, Guillet et al. [74] suggested that more than one motif may be capable of binding to a given restriction element. In addition, they found that a peptide of the cI protein shared a high degree of homology with I-

E^d, and bound strongly to it but did not elicit an I-E^d restricted T cell response. This suggested to them a model of antigen-Ia interaction in which foreign antigen (the external ligand) binds to Ia by displacing a sequence of the Ia molecule (the internal ligand) with which it shares some homology. The internal and external ligands were proposed to form the molecular basis of self-nonself discrimination and of alloreactivity.

PROJECT AND RATIONALE

A number of attempts have been made to elucidate the nature and mechanisms of antigen recognition by T lymphocytes, and much has been learned, particularly in the past five years. One approach has focused on the antigenic determinant seen by T cells with the purpose of describing its properties and mode of interaction with the T cell receptor and Ia. Two broad categories of antigen have been used: those derived from naturaly occurring proteins and random copolymers formed with a limited diversity of amino acids. This laboratory set out to design a synthetic antigen which would combine the advantages of both: a defined primary structure composed of only a few different amino acids. It was reasoned that an antigen of simple structure would be the most useful probe to examine T cell recognition. An additional feature was built in to this antigen: the sequence was chosen to produce a defined secondary structure.

The antigen poly-18, used in the present study, is a polymer of an 18 amino acid unit, EYK(EYA)$_5$, with an average m.w. of 11 000 [151]. Circular dichroism studies indicate an α-helical structure for this polymer [151]. The lysines in the polymer are separated spatially by 27 angstroms (five turns of a helix) and, therefore, line up on one face of the helix [151,152]. Immune responses to poly 18 were found to be under Ir gene control [152]. BALB/cCr (H-2^d) and DBA/2J (H-2^d) are high responders, whereas C57BL/10J (H-2^b) and C3H.HeJ (H-2^k) are nonresponders [152]. H-2^d mice were able to produce serum antibody and delayed-type hypersensitivty reactions to poly-18 [152]. Antigen-specific *in vitro* T cell proliferation could be elicited in T cells from immunized H-2^d mice, but not in H-2^b or H-2^k mice [153]. The minimum peptide sequence required for priming and triggering of these responses in BALB/c mice were also determined [153].

In the present project, poly 18-specific T cell hybridomas were generated from immunized BALB/cCr mice with the following objectives in mind (thesis chapters where these objectives are addressed are given in parentheses):

1. Analyse the poly 18-specific repertoire of T cells in these mice, by studying the fine specificity differences between the hybridomas generated, using substituted analogs of poly 18. (Chapter 2).

2. Select a sample of representative hybridomas and determine the contribution of antigen processing to the recognition of poly 18 and related synthetic peptides by these cells. (Chapters 3-6).

3. Determine the minimum length peptides required to stimulate the selected hybridomas and identify the critical residues of the antigen by substituting the amino acids at each position in the primary sequence. (Chapters 2, 4, and 6).

4. Identify the functional sites of the antigen which interact with the T cell receptor and Ia, using non-stimulatory substituted peptides to competitively inhibit antigen-specific activation of the selected hybridomas. (Chapter 5).

5. Examine the role of peptide secondary structure in stimulation of T cell hybridomas, using circular dichroism. (Chapters 3 and 5).

REFERENCES

1. **Gell, P.G.H., and B. Benacerraf.** 1959. Studies on hypersensitivity. II. Delayed hypersensitivity to denatured proteins in guinea pigs. *Immunology 2*:64.

2. **Thompson, K., M. Harris, E. Benjamini, G. Mitchell, and M. Noble.** 1972. Cellular and humoral immunity: A distinction in antigenic recognition. *Nature New Biol. 238*:20-21.

3. **Ishizaka, K., T. Kishimoto, G. Delepasse, and T.P. King.** 1974. Immunogenic properties of modified antigen E. I. Response of specific determinants for T cells in denatured antigen and polypeptide chains. *J.Immunol. 113*:70.

4. **Schirrmacher, V., and H. Wigzell.** 1974. Immune responses against native and chemically modified albumins in mice. II. Effect of alteration of electric charge and conformation on the humoral antibody response and helper T cell responses. *J.Immunol. 113*:1635.

5. **Berzofsky, J.A., A.N. Schechter, G.M. Shearer, and D.H. Sachs.** 1977. Genetic control of the immune response to staphylococcal nuclease. III. Time-course and correlation between the response to native nuclease and the response to polypeptide fragments. *J.Exp.Med. 145*:111.

6. **Corradin, G., and J.M. Chiller.** 1979. Lymphocyte specificity to protein antigens. II. Fine specificity of T cell activation with cytochrome c and derived peptides as antigenic probes. *J.Exp.Med. 149*:436.

7. **Maizels, R.M., J.A. Clarke, M.A. Harvey, A. Miller, and E.E. Sercarz.** 1980. Epitope specificity of the T cell proliferative response to lysozyme: proliferative T cells react predominantly to different determinants from those recognized by B cells. *Eur.J.Immunol. 10*:509.

8. **Sela, M.** 1969. Antigenicity: Some molecular aspects. *Science 166*:1365.

9. **Rosenstreich, D.L., and A.S. Rosenthal.** 1973. Peritoneal exudate lymphocyte. II. In vitro lymphocyte proliferation induced by brief exposure to antigen. *J.Immunol. 110*:934-942.

10. **Waldron, J.A., R.G. Horn, and A.S. Rosenthal.** 1973. Antigen-induced proliferation of guinea pig lymphocytes in vitro: obligatory role of macrophages in the recognition of antigen by immune T-lymphocytes. *J.Immunol. 111*:58-64.

11. **Rosenthal, A.S., and E.M. Shevach.** 1973. Function of macrophages in antigen recognition by guinea pig T lymphocytes. I. Requirement for histocompatible macrophages and lymphocytes. *J.Exp.Med. 138*:1194-1212.

12. **Shevach, E.M., and A.S. Rosenthal.** 1973. Function of macrophages in antigen recognition by guinea pig T lymphocytes. II. Role of the macrophage in the regulation of genetic control of the immune response. *J.Exp.Med. 138*:1213-1229.

13. **Rosenwasser, L.J., and A.S. Rosenthal.** 1978. Adherent cell function in murine T lymphocyte antigen recognition. II.Definition of genetically restricted and nonrestricted macrophage functions in T cell proliferation. *J.Immunol. 121*:2497-2501.

14. **Erb, P., and M. Feldmann.** 1975. The role of macrophages in the generation of T-helper cells. II.The genetic control of the macrophage-T-cell interaction for helper cell induction with soluble antigens. *J.Exp.Med. 142*:460-472.

15. **Yano, A., R.H. Schwartz, and W.E. Paul.** 1977. Antigen presentation in the murine T-lymphocyte proliferative response. I. Requirement for genetic identity at the major histocompatibility complex. *J.Exp.Med. 146*:828-843.

16. **Rosenthal, A.S., M.A. Barcinski, and J.T. Blake.** 1977. Determinant selection is a macrophage mediated immune response gene function. *Nature 267*:156.

17. **Barcinski, M.A., and A.S. Rosenthal.** 1977. Immune response gene control of determinant selection. I. Intramolecular mapping of the immunogenic sites on insulin recognized by guinea pig T and B cells. *J.Exp.Med. 145*:726.

18. **Unanue, E.R.** 1981. The regulatory role of macrophages in antigenic stimulation. Part two: symbiotic relationship between lymphocytes and macrophages. *Adv.Immunol. 31*:1-136.

19. **Ellner, J.J., and A.S. Rosenthal.** 1975. Quantitative and immunologic aspects of the handling of 2,4-dinitrophenyl guinea pig albumin by macrophages. *J.Immunol. 114*:1563-1569.

20. **Ziegler, K., and E.R. Unanue.** 1981. Identification of a macrophage antigen-processing event required for I-region-restricted antigen presentation to T lymphocytes. *J.Immunol. 127*:1869-1875.

21. **Watts, T.M., A.A. Brian, J.W. Kappler, P. Marrack, and H.M. McConnell.** 1984. Antigen presentation by supported planar membranes containing affinity-purified I-Ad. *Proc.Natl.Acad.Sci.USA 81*:7564-7568.

22. **Chesnut, R., R. Endres, and H.M. Grey.** 1980. Antigen recognition by T cells and B cells: recognition of cross-reactivity between native and denatured forms of globular antigens. *Clin.Immunol.Immunopathol. 15*:397.

23. **Shimonkevitz, R., J. Kappler, P. Marrack, and H. Grey.** 1983. Antigen recognition by H-2-restricted T cells. *J.Exp.Med. 158*:303-316.

24. **Shimonkevitz, R., S. Colon, J.W. Kappler, P. Marrack, and H.M. Grey.** 1984. Antigen recognition by H-2-restricted T cells. II. A tryptic ovalbumin peptide that substitutes for processed antigen. *J.Immunol. 133*:2067-2074.

25. **Allen, P.M., and E.R. Unanue.** 1984. Differential requirements for antigen processing by macrophages for lysozyme-specific T hybridomas. *J.Immunol. 132*:1077-1079.

26. **Streicher, H.I., I.J. Berkower, M. Busch, F.R.N. Gurd, and J.A. Berzofsky.** 1984. Antigen conformation determines processing requirements for T cell activation. *Proc.Natl.Acad.Sci.USA 81*:6831-6835.

27. **Kovac, Z., and R.H. Schwartz.** 1985. The molecular basis of the requirement for antigen processing of pigeon cytochrome c prior to T cell activation. *J.Immunol. 134*:3233-3240.

28. **Shastri, N., A. Miller, and E.E. Sercarz.** 1986. Amino acid residues distinct from the determinant region can profoundly affect activation of T cell clones by related antigens. *J.Immunol. 136*:371.

29. **Brian, A.A., and H.M. McConnell.** 1984. Allogeneic stimulation of cytotoxic T cells by supported planar membranes. *Proc.Natl.Acad.Sci.USA 81*:6159-6163.

30. **Watts, T.H., J. Gariepy, G. Schoolnik, and H.M. McConnell.** 1985. T cell activation by peptide antigen: Effect of peptide sequence and method of antigen presentation. *Proc.Natl.Acad.Sci.USA 82*:5480-5484.

31. **Chesnut, R.W., and H.M. Grey.** 1981. Studies on the capacity of B cells to serve as antigen-presenting cells. *J.Immunol. 126*:1075.

32. **McKean, D.J., A.J. Infante, A. Nilson, M. Kimoto, C.G. Fathman, E. Walker, and N. Warner.** 1981. Major histocompatibility complex-restricted antigen presentation to antigen-reactive T cells by B lymphocyte tumor cells. *J.Exp.Med. 154*:1419-1431.

33. **Chesnut, R.W., S.M. Colon, and H.M. Grey.** 1982. Antigen presentation by normal B cells, B cell tumors, and macrophages: functional and biochemical comparison. *J.Immunol. 128*:1764-1768.

34. **Lanzavecchia, A.** 1985. Antigen-specific interaction between T and B cells. *Nature 314*:537-539.

35. **Kakiuchi, T., R.W. Chesnut, and H.M. Grey.** 1983. B cells as antigen-presenting cells: the requirement for B cell activation. *J.Immunol. 131*:109-114.

36. **Chesnut, R.W., S.M. Colon, and H.M. Grey.** 1982. Requirements for the processing of antigens by antigen-presenting B cells. I. Functional comparison of B cell tumors and macrophages. *J.Immunol. 129*:2382-2388.

37. **Grey, H.M., S.M. Colon, and R.W. Chesnut.** 1982. Requirements for the processing of antigen by antigen-presenting B cells. II. Biochemical comparison of the fate of antigen in B cell tumors and macrophages. *J.Immunol. 129*:2389-2395.

38. **Glimcher, L., K.J. Kim, I. Green, and W.E. Paul.** 1982. Ia antigen-bearing B cell tumor lines can present protein antigen and alloantigen in a major histocompatibility complex-restricted fashion to antigen-reactive T cells. *J.Exp.Med. 155*:445.

39. **Kappler, J., J. White, D. Wegmann, E. Mustain, and P. Marrack.** 1982. Antigen presentation by Ia-positive B cell hybridomas to H-2-restricted T cell hybridomas. *Proc.Natl.Acad.Sci.USA 79*:3604.

40. **Ron, Y., and J. Sprent.** 1987. T cell priming in vivo: a major role for B cells in presenting antigen to T cells in lymph nodes. *J.Immunol. 138*:2848-2856.

41. **Rock, K.L., B. Benacerraf, and A.K. Abbas.** 1984. Antigen presentation by hapten-specific B lymphocytes. I. Role of surface immunoglobulin receptors. *J.Exp.Med. 160*:1102-1113.

42. **Ashwell, J.D., A.L. DeFranco, W.E. Paul, and R.H. Schwartz.** 1984. Antigen presentation by resting B cells. Radiosensitivity of the antigen-presentation function and two

dinstinct pathways of T cell activation. *J.Exp.Med. 159*:881-905.

43. **Gammon, G., N. Shastri, J. Cogswell, S. Wilbur, S. Sadegh-Nasseri, U. Krzych, A. Miller, and E. Sercarz.** 1987. The choice of T-cell epitopes utilized on a protein antigen depends on multiple factors distant from, as well as at the determinant site. *Immunol.Rev. 98*:53-73.

44. **Fox, B.S., F.R. Carbone, R.N. Germain, Y. Paterson, and R.H. Schwartz.** 1988. Processing of a minimal antigenic peptide alters its interaction with MHC molecules. *Nature 331*:538-540.

45. **Lorenz, R.G., and P.M. Allen.** 1988. Direct evidence for functional self-protein/Ia-molecule complexes in vivo. *Proc.Natl.Acad.Sci.USA 85*:5220-5223.

46. **Zinkernagel, R.M., and P.C. Doherty.** 1974. Restriction of in vitro T-cell mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semi-allogeneic system. *Nature 248*:701-702.

47. **Blanden, R.V., and G.L. Ada.** 1978. A dual recognition model of cytotoxic T cells based on thymic selection of precursors with low affinity for self-H-2 antigens. *Scand.J.Immunol. 7*:181-190.

48. **Janeway, C.A., Jr., H. Wigzell, and H. Binz.** 1976. Two different VH gene products make up the T cell receptors. *Scand.J.Immunol. 5*:993-1001.

49. **Zinkernagel, R.M., G.N. Callahan, A. Althage, S. Cooper, P.A. Klein, and J. Klein.** 1978. On the thymus in the differentiation of "H-2 self-recognition" by T cells: evidence for dual recognition?. *J.Exp.Med. 147*:882-896.

50. **Cohn, M., and R.E. Epstein.** 1978. T-cell inhibition of humoral responsiveness. Theory on the role of restrictive recognition in immune regulation. *Cell.Immunol. 39*:125-153.

51. **Langman, R.E.** 1978. Cell-mediated immunity and the major histocompatibility complex. *Rev.Physio.Biochem.Pharmacol. 81*:1-37.

52. **von Boehmer, H., W. Haas, and N.K. Jerne.** 1978. Major histocompatibility complex-linked immune-responsiveness is acquired by lymphocytes of low-responder mice differentiating in thymus of high responder mice. *Proc.Natl.Acad.Sci.USA 75*:2439-2442.

53. **Zinkernagel, R.M., and P.C. Doherty.** 1977. Major transplantation antigens, virus and specificity of surveillance T cells. The "altered self" hypothesis. *Contemp.Top.Immunobiol. 7*:179.

54. **Hunig, T.R., and M.J. Bevan.** 1981. Specificity of T-cell clones illustrates altered self hypothesis. *Nature 294*:460-462.

55. **Matzinger, P.** 1981. A one-receptor view of T-cell behaviour. *Nature 292*:497-501.

56. **Kappler, J.W., B. Skidmore, J. White, and P. Marrack.** 1981. Antigen-inducible, H-2 restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. *J.Exp.Med. 153*:1198-1214.

57. **Schwartz, R.H.** 1985. T lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. *Ann.Rev.Immunol. 3*:237-261.

58. **Heber-Katz, E., D. Hansburg, and R.H. Schwartz.** 1983. The Ia molecule of the antigen-presenting cell plays a critical role in immune response gene regulation of T cell activation. *J.Mol.Cell.Immunol. 1*:3-14.

59. **Werdelin, O.** 1982. Chemically related antigens compete for presentation by accessory cells to T cells. *J.Immunol. 129*:1883-1891.

60. **Rock, K.L., and B. Benacerraf.** 1983. Inhibition of antigen-specific T lymphocyte proliferation by structurally related Ir gene-controlled polymers. *J.Exp.Med. 157*:1618.

61. **Babbitt, B.P., P.M. Allen, G. Matsueda, E. Haber, and E.R. Unanue.** 1985. Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature 317*:359-361.

62. **Buus, S., S. Colon, C. Smith, J.H. Freed, C. Miles, and H.M. Grey.** 1986. Interaction between a "processed" ovalbumin peptide and Ia molecules. *Proc.Natl.Acad.Sci.USA 83*:3968-3971.

63. **Buus, S., A. Sette, S.M. Colon, D.M. Jenis, and H.M. Grey.** 1986. Isolation and characterization of antigen-Ia complexes involved in T cell recognition. *Cell 47*:1071-1077.

64. **Phillips, M.L., C.C. Yip, E.M. Shevach, and T.L. Delovitch.** 1986. Photoaffinity labeling demonstrates binding

between Ia molecules and nominal antigen on antigen-presenting cells. *Proc.Natl.Acad.Sci.USA 83*:5634-5638.

65. **Watts, T.H., H.E. Gaub, and H.M. McConnell.** 1986. T cell-mediated association of peptide antigen and major histocompatibility complex protein detected by energy transfer in an evanescent wavefield. *Nature 320*:176-179.

66. **Beck, B.N., P.A. Nelson, and C.G. Fathman.** 1983. The I-Ab mutant B6.C-H-2bm12 allows definition of multiple T cell epitopes on I-A molecules. *J.Exp.Med. 157*:1396.

67. **Sha, W.C., C.A. Nelson, R.D. Newberry, D.M. Kranz, J.H. Russell, and D.Y. Loh.** 1988. Selective expression of an antigen receptor on CD8-bearing T lymphocytes in transgenic mice. *Nature 335*:271-274.

68. **Clark, R.B., J. Chiba, S.E. Zweig, and E.M. Shevach.** 1982. T cell colonies recognize antigen in association with specific epitopes on Ia molecules. *Nature 295*:412-414.

69. **Frelinger, J.G., M. Shigeta, A.J. Infante, P.A. Nelson, M. Pierres, and C.G. Fathman.** 1984. Multiple functional sites on a single Ia molecule defined using T cell clones and antibodies with chain-determined specificity. *J.Exp.Med. 159*:704-715.

70. **Allen, P.M., D.J. McKean, B.N. Beck, J. Sheffield, and L.H. Glimcher.** 1985. Direct evidence that a class II molecule and a simple globular protein generate multiple determinants. *J.Exp.Med. 162*:1264-1274.

71. **Cease, K.B., I. Berkower, J. York-Jolley, and J.A. Berzofsky.** 1986. T cell clones specific for an amphipathic alpha-helical region of sperm whale myoglobin show differing fine specificities for synthetic peptides. *J.Exp.Med. 164*:1779-1784.

72. **Guillet, J.-G., M.-Z. Lai, T.J. Briner, J.A. Smith, and M.L. Gefter.** 1986. Interaction of peptide antigens and class II major histocompatibility complex antigens. *Nature 324*:260-262.

73. **Babbitt, B.P., G. Matsueda, E. Haber, E.R. Unanue, and P.M. Allen.** 1986. Antigenic competition at the level of peptide-Ia binding. *Proc.Natl.Acad.Sci.USA 83*:4509-4513.

74. **Guillet, J.-G., M.-Z. Lai, T.J. Briner, S. Buus, A. Sette, H.M. Grey, J.A. Smith, and M.L. Gefter.** 1987. Immunological self, nonself discrimination. *Science 235*:865-870.

75. **Buus, S., A. Sette, S.M. Colon, C. Miles, and H.M. Grey.** 1987. The relation between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunogenic peptides. *Science 235*:1353-1358.

76. **Adorini, L., A. Sette, S. Buus, H.M. Grey, M. Darsley, P.V. Lehmann, G. Doria, Z.A. Nagy, and E. Appella.** 1988. Interaction of an immunodominant epitope with Ia molecules in T-cell activation. *Proc.Natl.Acad.Sci.USA 85*:5181-5185.

77. **Bjorkman, P.J., M.A. Saper, B. Samraoui, W.S. Bennett, J.L. Strominger, and D.C. Wiley.** 1987. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature 329*:506-512.

78. **Bjorkman, P.J., M.A. Saper, B. Samraoui, W.S. Bennett, J.L. Strominger, and D.C. Wiley.** 1987. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature 329*:512-518.

79. **Brown, J.H., ,T. Jardetzky, M.A. Saper, B. Samraoui, P.J. Bjorkman, and D.C. Wiley.** 1988. A hypothetical model of the foreign antigen binding site of Class II histocompatibility molecules. *Nature 332*:845-850.

80. **Allison, J.P., B.W. McIntyre, and D. Bloch.** 1982. Tumor-specific antigen of murine T lymphoma defined with monoclonal antibody. *J.Immunol. 129*:2293-2300.

81. **Bigler, R.D., D.E. Fisher, C.Y. Wang, E.A. Rinnooykan, and H.G. Kunkel.** 1983. Idiotype-like molecules on cells of a human T cell leukemia. *J.Exp.Med. 158*:1000-1005.

82. **Haskins, K., R. Kubo, J. White, M. Pigeon, J. Kappler, and P. Marrack.** 1983. The major histocompatibility complex-restricted antigen receptor on T cells. I.Isolation with a monoclonal antibody. *J.Exp.Med. 157*:1149-1169.

83. **Meuer, S., K. Fitzgerald, R. Hussey, J. Hodgdon, S. Schlossman, and E. Reinherz.** 1983. Clonotypic structures involved in antigen-specific human T cell function. Relationship to the T3 molecular complex. *J.Exp.Med. 157*:705-719.

84. **Samelson, L.E., and R.H. Schwartz.** 1984. Characterization of the antigen-specific T cell receptor from a pigeon citochrome c-specific T cell hybrid. *Immunol.Rev. 81*:131-144.

85. **Staerz, U.D., M.S. Pasternack, J.R. Klein, J.D. Benedetto, and M.J. Bevan.** 1984. Monoclonal antibodies specific for a murine cytotoxic T-lymphocyte clone. *Proc.Natl.Acad.Sci.USA 81*:1799-1803.

86. **Kaye, J., S. Procelli, J. Tite, B. Jones, and C. Janeway,Jr..** 1983. Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen and antigen-presenting cells in the activation of T cells. *J.Exp.Med. 158*:836-856.

87. **Lancki, D.W., M.I. Lorber, M.R. Loken, and F.W. Fitch.** 1983. A clone specific antibody that inhibits cytolysis of a cytolytic T-cell clone. *J.Exp.Med. 157*:921-935.

88. **Reinherz, E., S. Meuer, K. Fitzgerald, R. Hussey, J. Hodgdon, O. Acuto, and S. Schlossman.** 1983. Comparison of T3-associated 49- and 42-kilodalton cell surface molecules on individual human T-cell clones: evidence for peptide variability in T-cell receptor structures. *Proc.Natl.Acad.Sci.USA 80*:4104-4108.

89. **Goding, J.W., and A.W. Harris.** 1981. Subunit structure of cell surface proteins: disulfide bonding in antigen receptors, Ly-2/3 antigens, and transferrin receptors of murine T and B lymphocytes. *Proc.Natl.Acad.Sci.USA 78*:4530-4534.

90. **McIntyre, B.W., and J.P. Allison.** 1983. The mouse T cell receptor: structural heterogeneity of molecules of normal T cells defined by xenoantiserum. *Cell 34*:739-746.

91. **Kappler, J., R. Kubo, K. Haskins, C. Hannum, P. Marrack, M. Pigeon, B.W. McIntyre, J.P. Allison, and I. Trowbridge.** 1983. The major histocompatibility complex-restricted antigen receptor on T cells in mouse and man: identification of constant and variable peptides. *Cell 35*:295-302.

92. **Allison, J.P., L. Ridge, J. Lund, J. Gross-Pelose, L.L. Lanier, and B.W. McIntyre.** 1984. The murine T cell antigen receptor and associated structures. *Immunol.Rev. 81*:145-160.

93. **Acuto, O., S.C. Meuer, J.C. Hodgdon, S.F. Schlossman, and E.L. Reinherz.** 1983. Peptide variability exists within the alpha and beta subunits of the T cell receptor for antigen. *J.Exp.Med. 158*:1368.

94. **Marrack, P., R. Shimonkevitz, C. Hannum, K. Haskins, and J. Kappler.** 1983. The major histocompatibility complex-restricted antigen receptor on T cells. IV.An antiidiotypic antibody predicts both antigen and I-specificity. *J.Exp.Med. 158*:1635-1646.

95. **Samelson, L.E.** 1985. An analysis of the structure of the antigen receptor on a pigeon cytochrome c-specific T cell hybrid. *J.Immunol. 134*:2529-2535.

96. **Hedrick, S.M., D.I. Cohen, E.A. Nielsen, and M.M. Davis.** 1984. Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature 308*:149-153.

97. **Yanagi, Y., Y. Yoshikai, K. Leggett, S.P. Clark, I. Aleksander, and T.W. Mak.** 1984. A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. *Nature 308*:145-149.

98. **Acuto, O., M. Fabbi, J. Smart, C.B. Poole, J. Protentis, H.D. Royer, S.F. Schlossman, and E.L. Reinherz.** 1984. Purification and NH2-terminal amino acid sequencing of the beta subunit of a human T-cell antigen receptor. *Proc.Natl.Acad.Sci.USA 81*:3851-3855.

99. **Chien, Y.-h., D.M. Becker, T. Lindsten, M. Okamura, D.I. Cohen, and M.M. Davis.** 1984. A third type of murine T-cell receptor gene. *Nature 312*:31-35.

100. **Saito, H., D.M. Kranz, Y. Takagaki, A.C. Hayday, H.N. Eisen, and S. Tonegawa.** 1984. A third rearranged and expressed gene in a clone of cytotoxic T lymphocytes. *Nature 312*:36-40.

101. **Saito, H., D.M. Kranz, Y. Takagaki, A.C. Hayday, H.N. Eisen, and S. Tonegawa.** 1984. Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences. *Nature 309*:757-762.

102. **Chien, Y., M. Iwashima, K.B. Kaplan, J.F. Elliott, and M.M. Davis.** 1987. A new T-cell receptor gene located within the alpha locus and expressed early in T-cell differentiation. *Nature 327*:677-682.

103. **Brenner, M.B., J. McLean, D.P. Dialynas, J.L. Strominger, J.A. Smith, F.L. Owen, J.G. Seidman, S. Ip, F. Rosen, and M.S. Krangel.** 1986. Identification of a putative second T-cell receptor. *Nature 322*:145-149.

104. **Bank, I., R.A. DePinho, M.B. Brenner, J. Cassimeris, F.W. Alt, and L. Chess.** 1986. A functional

T3 molecule associated with a novel heterodimer on the surface of immature human thymocytes. *Nature 322*:179-181.

105. **Bluestone, J.A., D. Pardoll, S.O. Sharrow, and B.J. Fowlkes.** 1987. Cnaracterization of murine thymocytes with CD-3-associated T-cell receptor structures. *Nature 326*:82-84.

106. **Pardoll, D.M., B.J. Fowlkes, J.A. Bluestone, A. Kruisbeek, W.L. Maloy, J.E. Coligan, and R.H. Schwartz.** 1987. Differential expression of two distinct T-cell receptors during thymocyte development. *Nature 326*:79-81.

107. **Stingl, G., F. Koning, H. Yamada, W.M. Yokoyama, E. Tschachler, J.A. Bluestone, G. Steiner, L.E. Samelson, A.M. Lew, J.E. Coligan, and E.M. Shevach.** 1987. Thy-1+ dendritic epidermal cells express T3 antigen and the T-cell receptor gamma chain. *Proc.Natl.Acad.Sci.USA 84*:4586-4590.

108. **Yancopoulos, G.D., T.K. Blackwell, H. Suh, L. Hood, and F.W. Alt.** 1986. Introduced T cell receptor variable region gene segments recombine in pre-B cells: evidence that B and T cells use a common recombinase. *Cell 44*:251-259.

109. **Fujimoto, S., and H. Yamagishi.** 1987. Isolation of an excision product of T-cell receptor alpha chain gene rearrangements. *Nature 327*:242-243.

110. **Lee, N.E., and M.M. Davis.** 1988. T cell receptor beta chain genes in BW5147 and other AKR tumors. Deletion order of murine V beta gene segments and possible 5' regulatory regions. *J.Immunol. 140*:1665-1675.

111. **Malissen, M., C. McCoy, D. Blanc, J. Trucy, C. Devaux, A.-M. Schmitt-Verhulst, F. Fitch, L. Hood, and B. Malissen.** 1986. Direct evidence for chromosomal inversion during T-cell receptor beta gene rearrangements. *Nature 319*:28-33.

112. **Kronenberg, M., J. Goverman, R. Haars, M. Malissen, E. Kraig, L. Phillips, T. Delovitch, N. Suciu-Foca, and L. Hood.** 1985. Rearrangement and transcription of the beta chain genes of the T-cell antigen receptor in different types of murine lymphocytes. *Nature 313*:647-653.

113. **Chien, Y., M. Iwashima, D.A. Wettstein, K.B. Kaplan, J.F. Elliott, W. Born, and M.M. Davis.** 1987. T-

cell receptor delta gene rearrangements in early thymocytes. *Nature 330*:722-727.

114. **Elliott, J.F., E.P. Rock, P.A. Patten, M.M. Davis, and Y. Chien.** 1988. The adult T-cell receptor delta chain is diverse and distinct from that of fetal thymocytes. *Nature 331*:627-631.

115. **Hayday, A.C., H. Saito, S.D. Gillies, D.M. Kranz, G. Tanigawa, H.N. Eisen, and S. Tonegawa.** 1985. Structure, organisation, and somatic rearrangement of T cell gamma genes. *Cell 40*:259-269.

116. **Davis, M.M., and P.J. Bjorkman.** 1988. T-cell antigen receptor genes and T-cell recognition. *Nature 334*:395-402.

117. **Yancopoulos, G.D., and F.W. Alt.** 1986. Regulation of the assembly and expression of variable-region genes. *Ann.Rev.Immunol. 4*:339-368.

118. **Siu, G., M. Kronenberg, E. Strauss, R. Haars, T.W. Mak, and L. Hood.** 1984. The structure, rearrangement and expression of D beta gene segments of the murine T-cell antigen receptor. *Nature 311*:344-350.

119. **Kronenberg, M., G. Siu, L. Hood, and N. Shastri.** 1986. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. *Ann.Rev.Immunol. 4*:529-591.

120. **Patten, P., T. Yokota, J. Rothbard, Y. Chien, K.-I. Arai, and M.M. Davis.** 1984. Structure, expression and divergence of T-cell receptor beta chain variable regions. *Nature 312*:40-46.

121. **Barth, R., B. Kim, N. Lan, T. Hunkapiller, N. Sobieck, A. Winoto, H. Gershenfeld, C. Okada, D. Hansburg, I. Weissman, and L. Hood.** 1985. The murine T-cell receptor employs a limited repertoire of expressed V beta gene segments. *Nature 316*:517-523.

122. **Davies, D.R., and H. Metzger.** 1983. Structural basis of antibody function. *Ann.Rev.Immunol. 1*:87-117.

123. **Alzari, P.M., M.-B. Lascombe, and R.J. Poljak.** 1988. Three-dimensional structure of antibodies. *Ann.Rev.Immunol. 6*:555-580.

124. **Dembic, Z., W. Haas, S. Weiss, J. McCubrey, H. Kiefer, H. von Boehmer, and M. Steinmetz.** 1986. Transfer of specificity by murine alpha and beta T-cell receptor genes. *Nature 320*:232-238.

125. **Saito, T., A. Weiss, J. Miller, M.A. Norcross, and R.N. Germain.** 1987. Specific antigen-Ia activation of transfected human T cells expressing murine Ti alpha-beta-human T3 receptor complexes. *Nature 325*:125-130.

126. **Fink, P.J., L.A. Matis, D.L. McElligott, M. Bookman, and S.M. Hedrick.** 1986. Correlations between T-cell specificity and the structure of the antigen receptor. *Nature 321*:219-226.

127. **Kappler, J.W., T. Wade, J. White, E. Kushnir, M. Blackman, J. Bill, N. Roehm, and P. Marrack.** 1987. A T cell receptor V beta segment that imparts reactivity to a Class II major histocompatibility complex product. *Cell 49*:263-271.

128. **Goverman, J., K. Minard, N. Shastri, T. Hunkapiller, D. Hansburg, E. Sercarz, and L. Hood.** 1985. Rearranged beta T cell receptor genes in a helper T cell clone specific for lysozyme: no correlation between V beta and MHC restriction. *Cell 40*:859-867.

129. **Winoto, A., J.L. Urban, N.C. Lan, J. Goverman, L. Hood, and D. Hansburg.** 1986. Predominant use of a V(alpha) gene segment in mouse T-cell receptors for cytochrome c. *Nature 324*:679-682.

130. **Hedrick, S.M., I. Engel, D.L. McElligott, P.J. Fink, M. Hsu, D. Hansburg, and L.A. Matis.** 1988. Selection of amino acid sequences in the beta chain of the T cell antigen receptor. *Science 239*:1541-1544.

131. **Thomas, J.W., W. Danho, E. Bullesbach, J. Fohles, and A.S. Rosenthal.** 1981. Immune response gene control of determinant selection. III. Polypeptide fragments of insulin are differentially recognized by T but not by B cells in insulin-immune guinea pigs. *J.Immunol. 126*:1095.

132. **Solinger, A.M., M.E. Ultee, E. Margoliash, and R.H. Schwartz.** 1979. The T lymphocyte proliferative response to cytochrome c. I. Demonstration of a T cell heteroclitic proliferative response and identification of a single topographic antigenic determinant on pigeon cytochrome c whose immune recognition requires two complementing major histocompatibility complex linked response genes. *J.Exp.Med. 150*:830.

133. **Adorini, L., M.A. Harvey, A. Miller, and E.E. Sercarz.** 1979. Fine specificity of regulatory T cells. II. Suppressor and helper T cells are induced by different regions of hen egg lysozyme in a genetically non-responder mouse strain. *J.Exp.Med. 150*:293.

134. **Katz, M.E., R.M. Maizels, L. Wicker, A. Miller, and E.E. Sercarz.** 1982. Immunological focusing by the mouse major histocompatibility complex: Mouse strains confronted with distantly related lysozymes confine their attention to very few epitopes. *Eur.J.Immunol. 12*:535.

135. **Allen, P.M., D.J. Strydom, and E.R. Unanue.** 1984. Processing of lysozyme by macrophages: identification of the determinant recognized by two T-cell hybridomas. *Proc.Natl.Acad.Sci.USA 81*:2489-2493.

136. **Infante, A.J., M.Z. Atassi, and C.G. Fathman.** 1981. T cell clones reactive with sperm whale myoglobin. Isolation of clones with specificity for individual determinants on myoglobin. *J.Exp.Med. 154*:1342.

137. **Berkower, I., G. Buckenmeyer, F.R.N. Gurd, and J.A. Berzofsky.** 1982. A possible immunodominant epitope recognized by murine T lymphocytes immune to different myoglobins. *Proc.Natl.Acad.Sci.USA 79*:4723.

138. **Berkower, I., L.A. Matis, G.K. Buckenmeyer, F.R.N. Gurd, D.L. Longo, and J.A. Berzofsky.** 1984. Identification of distinct predominant epitopes recognized by myoglobin-specific T cells under the control of different Ir genes and characterization of representative T cell clones. *J.Immunol. 132*:1370.

139. **Townsend, A.R.M., J. Rothbard, F.M. Gotch, G. Bahadur, D. Wraith, and A.J. McMichael.** 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. *Cell 44*:959-968.

140. **Hackett, C.J., J.L. Hurwitz, B. Dietzschold, and W. Gerhard.** 1985. A synthetic decapeptide of influenza virus hemagglutinin elicits helper T cells with the same fine recognition specificities as occur in response to whole virus. *J.Immunol. 135*:1391-1394.

141. **Berkower, I., H. Kawamura, L.A. Matis, and J.A. Berzofsky.** 1985. T cell clones to two major T cell epitopes of myoglobin: effect of I-A/I-E restriction on epitope dominance. *J.Immunol. 135*:2628-2634.

142. **Hansburg, D., T. Fairwell, R.H. Schwartz, and E. Appella.** 1983. The T lymphocyte response to cytochrome c. IV. Distinguishable sites on a peptide antigen which affect antigenic strength and memory. *J.Immunol. 131*:319.

143. **Hansburg, D., E. Heber-Katz, T. Fairwell, and E. Appella.** 1983. Major histocompatibility complex-controlled, antigen-presenting cell-expressed specificity of T cell antigen recognition. Identification of a site of interaction and its relationship to Ir genes. *J.Exp.Med. 158*:25-39.

144. **Allen, P.M., G.R. Matsueda, R.J. Evans, J.B.Jr. Dunbar, G.R. Marshall, and E.R. Unanue.** 1987. Identification of the T-cell and Ia contact residues of a T-cell antigenic epitope. *Nature 327*:713-715.

145. **Sette, A., S. Buus, S. Colon, J.A. Smith, C. Miles, and H.M. Grey.** 1987. Structural characteristics of an antigen required for its interaction with Ia and recognition by T cells. *Nature 328*:395-399.

146. **Pincus, M.R., F. Gerewitz, R.H. Schwartz, and H.A. Scheraga.** 1983. Correlation between the conformation of cytochrome c peptides and their stimulatory activity in a T-lymphocyte proliferation assay. *Proc.Natl.Acad.Sci.USA 80*:3297-3300.

147. **DeLisi, C., and J.A. Berzofsky.** 1985. T-cell antigenic sites tend to be amphipathic structures. *Proc.Natl.Acad.Sci.USA 82*:7048-7052.

148. **Rothbard, J.B.** 1986. Peptides and the cellular immune response. *Ann.Inst.Pasteur 137E*:518-528.

149. **Rothbard, J.B., and W.R. Taylor.** 1988. A sequence pattern common to T cell epitopes. *EMBO J. 7*:93-100.

150. **Rothbard, J.B., R.I. Lechler, K. Howland, V. Bal, D.D. Eckels, R. Sekaly, E.O. Long, W.R. Taylor, and J.R. Lamb.** 1988. Structural model of HLA-DR1 restricted T cell antigen recognition. *Cell 52*:515-523.

151. **Barton, M., B. Singh, and E. Fraga.** 1977. Synthetic polypeptide antigens of defined geometry. *J.Am.Chem.Soc. 99*:8491-8498.

152. **Singh, B., E. Fraga, and M. Barton.** 1978. Characterization and genetic control of the immune response to synthetic polypeptide antigens of defined geometry. *J.Immunol. 121*:784-789.

153. **Singh, B., K.-C. Lee, E. Fraga, A. Wilkinson, M. Wong, and M. Barton.** 1980. Minimum peptide sequences necessary for priming and triggering of humoral and cell mediated immune responses in mice: use of synthetic peptide antigens of defined structure. *J.Immunol. 124*:1336-1343.

CHAPTER 2

Fine specificity of antigen recognition by T cell hybridoma clones specific for poly-18[1]

The immune reactivity of T lymphocytes involves the concomitant specific recognition of molecular variants on both foreign antigens and molecules encoded by the genes of the major histocompatibility complex (MHC). Several peptide and protein antigens have been closely examined to define the fine specificity of T cell recognition and, in each case, T cells can discriminate between one or several amino acid substitutions in the antigen or the MHC gene product [1-14]. We have been working with synthetic polypeptide antigens of defined sequence and conformation [15] to analyze T cell fine specificity. On the one hand, these antigens are simpler in sequence than most globular proteins; on the other hand, they avoid the problem of heterogeneity in molecular nature and immunogenicity of random copolymers of amino acids [16,17]. Because protein antigens are normally processed by antigen-presenting cells (APC) before being recognized by T cells,

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synthetic peptide antigens of defined sequence offer a simpler system to investigate this phenomenon.

In this report, we have used the synthetic polypeptide poly 18, poly EYK(EYA)$_5$, to analyze the specific repertoire of BALB/cCr mice to this antigen. This was achieved with cloned T cell hybridomas made by fusion of poly-18-reactive T cell blasts with the BW5147 thymoma. We have identified two T cell clonotypes in the BALB/cCr response to poly-18. These hybridomas are IA^d-restricted and release interleukin 2 (IL-2) in response to poly-18. Our ability to rapidly synthesize related peptide antigens has allowed for a rigorous definition of T cell fine specificity. We have also determined the minimum peptide sequence that can activate these T hybridomas.

MATERIALS AND METHODS

Animals. Mice of strains BALB/cCr, C3H.H3J, C57BL/10J, DBA/2J, and CBA/J were bred at the Ellerslie Animal Farm of the University of Alberta, or were purchased from The Jackson Laboratory, Bar Harbor, ME.

Antigens. The synthetic strategy for poly-18 and the various related peptides was as described previously [15]. In brief, it utilized a fragment condensation scheme with selectively deprotected amino and carboxyl components, using the dicyclohexyl-carbodiimidehydroxybenzotriazole method. The protecting groups used were tert-butyl ester for the glutamic acid side chain group, 2-(4-biphenyl)isopropoxycarbonyl for the amino terminus, and phenyl ester for the carboxyl terminus. Protected peptides were purified by Sephadex LH-20 gel filtration in DMF. Amino acid analysis and thin-layer chromatography were performed at every stage of coupling to monitor for the completion of reactions. Final deprotections were performed with HBr in TFA as described [15]. Peptides were dissolved in saline and were adjusted to pH 7.2 with 0.1 N NaOH. Table 2.1 lists the synthetic antigens used in this study. Peptide solutions were sterilized by filtration through a 0.22 μm filter.

T cell proliferation assays. T cell proliferation assays were performed as previously described [18]. In brief, BALB/cCr mice were immunized with 25 μg poly-18 in

complete Freund's adjuvant (CFA) per hind foot pad. Popliteal lymph nodes were excised, and lymph node cells (5 x 10^5 cells/mL) were incubated with appropriate doses of antigen and irradiated (4000 rad) BALB/cCr spleen cells (5 x 10^6 cells/mL) for 4 days in RPMI 1640 containing 10 mM HEPES and 10% heat-inactivated horse serum. Incorporation of [^{3}H]thymidine, by pulsing for 24 h, was used as an index of proliferation.

T cell lines. T cell lines were generated as previously described [19], with minor modifications. Lymph node cells (10^5 cells/mL) from poly-18/CFA-primed BALB/cCr mice were incubated with 50 μg/mL antigen and irradiated (4000 rad) BALB/cCr spleen cells (2.5 x 10^6 cells/mL) in RPMI 1640 containing 10 mM HEPES and 10% heat-inactivated horse serum for 8 days. Viable cells were separated by centrifugation on a Lympholyte M cushion (Cedarlane Laboratories, Hornby, Ontario, Canada). Viable cells obtained from the interface were washed and recultured in medium containing RPMI 1640, 10 mM HEPES, 1 mM glutamine, 5 x 10^{-5} M 2-mercaptoethanol, 10% heat-inactivated fetal calf serum, and 1 U/mL penicillin-streptomycin (Gibco). T cells (10^5 cells/mL) were cultured for 8 days in the presence of 100 μg/mL of antigen and irradiated (4000 rad) BALB/cCr spleen cells (2.5 x 10^6 cells/mL). The cycle of separating viable T cells with the addition of fresh APC and antigen was repeated every 8 days. Proliferation assays on the T cell lines were done by

incubating 10^4 or 10^5 T cells with 10^6 irradiated (4000 rad) BALB/cCr spleen cells and varying amounts of antigen for 2 to 3 days. Incorporation of [^{3}H]thymidine, by pulsing for 24 h, was used as an index of proliferation.

T cell hybridomas. T cell hybridomas were generated according to established procedures [20], with minor modifications. After an 8-day culture cycle, T cell lines were centrifuged on Lympholyte M. Viable T cells (10^5 cells/mL) were cultured with irradiated (4000 rad) spleen cells (2.5 x 10^6 cells/mL), 100 μg/mL of poly-18, and 1% IL 2-containing EL4 supernatant for 4 days. After this time, the viable cells were obtained by centrifugation on lympholyte M, and were fused with BW5147 in a 1:1 ratio. Hybridomas were selected in the presence of hypoxanthine, thymidine, aminopterin, and ouabain. Mouse red blood cells were used as filler cells. The wells scored positive for growth were assayed for IL 2 production in the presence of poly-18 and BALB/cCr APC as described below. Hybridomas selected for antigen/APC-induced IL 2 production were subcloned by limiting dilution.

IL 2 assay. T hybridoma cells (10^4 or 10^5) were co-cultured with 10^6 irradiated (4000 rad) spleen cells in the presence or absence of antigen in 200 μL of culture medium. After 2 days, supernatants were collected and were assayed for IL 2 content in a secondary culture by using either NK11D1 cells [21] or thymocytes [22]. Cells (10^4 NK11D1 or

10^5 thymocytes) were cultured for 48 h (NK11D1) or 4 days (thymocytes) with serial dilutions of primary supernatant (starting at 25% primary supernatant), and the degree of stimulation was measured by the incorporation of [^{3}H]thymidine (1 μCi/well). IL 2 units were calculated according to the method of Hedrick *et al.* [23], using a computer program devised by B.S. Davis (Immunex Corp., Seattle, WA) and kindly provided to us. Various peptides and APC of different genotypes were used in a similar manner to determine their effect on the IL 2 production by these hybridomas.

Monoclonal antibodies. MKD6, anti-IA^d [20] and 10.3.6 anti-IA^k [24] monoclonal antibodies were used after ammonium sulfate precipitation of ascites followed by dialysis of the solubilized precipitate. Antibodies were sterilized by filtration through a 0.22 μm filter and were used at the dilutions described in the text.

RESULTS

Specificity of poly-18-reactive T cell lines and hybridomas. T cells obtained from the popliteal lymph nodes of poly-18/CFA-immunized BALB/cCr mice were used to generate poly-18-specific T cell lines. As shown in Table 2.2, these T cells were specific for poly-18 and did not react with other protein antigens, including purified protein derivative (PPD), to which they were reactive when the T cell line cultures were set up. T cell hybridomas were made by fusion of T cell blasts, obtained from T cell lines, with the BW5147 thymoma, as described. The hybridomas were screened for their ability to release IL 2 with poly-18 in the presence of BALB/cCr APC. The positive wells were cloned, by limiting dilution, and were analyzed for fine specificity.

One group of hybridomas released IL 2 when poly-18 was presented by H-2^d APC, but not when TNP-poly-18 or poly-EYA were used as antigens (Table 2.3). This suggested strongly that the lysine side chains were critical for activation (Table 2.1). This group of hybridomas was designated Group A. The other group of hybridomas released IL 2 when either poly-18 or poly-EYA were presented by H-2^d APC (Table 2.3). This finding suggested that the lysine residues were not involved in the poly-18/H-2^d-induced activation of this set of hybridomas. The second group was designated Group B.

MHC restriction of T cell hybridomas. The results presented in Figure 1A show that A.1.1, a Group A anti-poly-18 T cell hybridoma, released IL 2 only when poly-18 was presented by H-2^d APC, but not by H-2^b or H-2^k APC. Anti-IA^d monoclonal antibody MKD6 blocked activation, whereas 10.3.6 (anti-IA^k monoclonal antibody) did not block activation of these hybridomas. Similar results (data not shown) were obtained with the other T cell hybridomas listed in Group A. This suggests that these hybridomas are IA^d-restricted and are not alloreactive to H-2^b or H-2^k (Fig. 1A). The results for Group B hybridomas were similar. The data in Figure 1B show that T cell hybridoma B.1.1 is IA^d-restricted and is not alloreactive to H-2^b or H-2^k. Similar results (data not shown) were obtained with other Group B T cell hybridomas, thus indicating that both Group A and Group B T cell hybridomas, although reactive to distinct determinants on poly-18, are restricted to the same MHC element, I-A^d.

Fine specificity of Group A hybridomas. Lysine residues in poly-18, as shown above, are critical for activation of this group of T cell hybridomas. The repeating 18 amino acid unit in poly-18, $EYK(EYA)_5$, also called "monomer 18", containing one lysine residue, did not activate this group of hybridomas (Table 2.4) while a synthetic dimer, "dimer 18", made of two "monomer 18" units coupled head to tail, could elicit a response (Table 2.4). This suggested that the sequential recognition of two lysines in the antigen was important. Two

more peptides, 18-QIK and EYK(EYA)$_5$EYK, were synthesized to further confirm these observations. In both peptides, a tripeptide was coupled to the carboxyl end of the "monomer 18", Gln-Ile-Lys in 18-QIK and Glu-Tyr-Lys in EYK(EYA)$_5$EYK. Both of these peptides were able to activate (Table 2.4). The fact that the only common extra amino acid in 18-QIK and EYK(EYA)$_5$EYK is the carboxyl end lysine group, as compared to the monomer, suggested that this lysine was playing a role in activation of Group A T cell hybridomas.

These results identified Glu-Tyr-Lys-(Glu-Tyr-Ala)$_5$-Glu-Tyr-Lys as the sequence sufficient to activate these hybridomas. We next asked if we could further reduce the number of intervening amino acid residues between the two lysines and still retain activity. Therefore, a series of peptides, EYK(EYA)$_5$EYK, EYK(EYA)$_4$EYK, EYK(EYA)$_3$EYK, and EYK(EYA)$_2$EYK, were synthesized. The smallest peptide capable of activation of Group A hybridomas was found to be EYK(EYA)$_3$EYK (Table 2.4). Because the peptide (EYA)$_4$EYK was inactive (Table 2.4), and the only difference between it and the active analog EYK(EYA)$_3$EYK was an alanine instead of a lysine residue at position 3, it appeared that the presence of lysine at this position was mandatory for activation. (The residues in the minimal sequence Glu-Tyr-Lys-(Glu-Tyr-Ala)$_3$-Glu-Tyr-Lys have been arbitrarily numbered from the N-terminal end as 1 to 15.) These data provide direct evidence

for the involvement of lysine 3 and lysine 15 in the activation of this group of hybridomas.

Finally, the inability of poly-(EYKEYA) to activate (Table 2.4) suggested an important site at position 9 where the presence of an alanine residue may be critical for activation. In summary, Group A hybridomas are I-A^d-restricted and require a minimum peptide sequence of 15 amino acids for activation. The lysine at position 3 and the alanine at position 9 were found to be critical for activation of this group of T cell hybrids. Based on these data, the lysine at position 15 also appeared to be critical. However, subsequent experiments revealed that substitutions of this residue did not critically affect reactivity of the antigen (chapter 4).

Fine specificity of Group B hybridomas. This group of hybridomas did not require the lysine residues present in poly-18 for activation. This was in sharp contrast to the hybridomas in Group A. Thus, predictably, poly-18, poly-(EYA), poly-(EFA), and "monomer 18" activated, but poly-(KYA) did not (Table 2.5). Because poly-(EYA) could activate, these results suggested that the active portion of the "monomer 18" which could activate these hybridomas was $(Glu\text{-}Tyr\text{-}Ala)_5$. The inability of poly-(KYA) to activate suggested the involvement of one or more of the five glutamic acid residues in $(Glu\text{-}Tyr\text{-}Ala)_5$. The involvement of one or more tyrosine residues was suggested by the depressed ability of

poly-(EFA) to activate this group. The suggested involvement of glutamic acid and tyrosine residues needs to be investigated further.

To determine the minimum peptide needed to activate Group B hybridomas, we used the peptides EYK(EYA)$_5$EYK, EYK(EYA)$_4$EYK, EYK(EYA)$_3$EYK, EYK(EYA)$_2$EYK, (EYA)$_4$, and (EYA)$_4$EYK. Because (EYA)$_4$ was inactive (Table 2.5), the minimum active peptide sequence was probably longer than 12 amino acids. EYK(EYA)$_5$EYK and EYK(EYA)$_4$EYK were active (Table 2.5), thus suggesting that the length of the active peptide was between 12 and 18 amino acid residues long. The ability of (EYA)$_4$EYK to activate (Table 2.5) and the inability of the related peptide EYK(EYA)$_3$EYK to activate led us to assign as the minimal length the 15 amino acid peptide Glu-Tyr-Ala-(Glu-Tyr-Ala)$_3$-Glu-Tyr-Lys/Ala. (The residues in this peptide have been arbitrarily numbered 1 to 15 from the N-terminal glutamic acid.) The ability of poly-EYA, poly-18, monomer-18, and (EYA)$_4$EYK to activate group B hybridomas suggests that it does not matter whether the last C-terminal amino acid is alanine or lysine. Thus, we have demonstrated that both Group A and Group B hybridomas are activated by 15 amino acid-long minimal sequences.

DISCUSSION

The discovery of MHC restriction led to the concept that foreign antigens must be seen by the T cell receptor in the context of self-MHC gene products. The ability of nonresponder APC to present antigen has been used to explain this phenomenon as T cell repertoire defects, with the low responder strains not having the relevant T cells [23,25-28]. In contrast, investigations in guinea pigs by using heterologous insulins have suggested determinant selection by Ia antigens as an explanation for the same phenomenon [1,29,30]. These results indicated that strain 13 "Ia" could interact functionally with the B chain of pork insulin, whereas strain 2 "Ia" could not [29]. More recently, the ability of high responder B10.A APC, in contrast to the inability of low responder B10.A(5R) APC, to present pigeon cytochrome *c* to B10.A(5R)-primed T cells has been interpreted to suggest that Ia antigens from the high responder strain can functionally interact with pigeon cytochrome *c*, whereas the low responder Ia molecules cannot [31,32].

These apparently contradictory explanations for essentially the same phenomenon have been integrated in the trimolecular complex proposed by Heber-Katz *et al.* [32]. This model clearly identifies three interactions: antigen-Ia, antigen-TCR, and Ia-TCR. Cytochrome *c* has been the

antigen system used rigorously to support this model because cytochrome *c*-specific T cell clones have been generated which show degeneracy in their Ia restriction specificity [23]. Lys-99 in cytochrome *c* has been identified as the contact site in the antigen-TCR interaction, and Ala-103 has been suggested to be the putative contact site in the antigen-Ia interaction, in the B10.A T cell response to the 81-104 pigeon cytochrome *c* fragment [31-33]. The presence of an agretope (an Ia interaction site on the antigen) has also been suggested in the H-2^b response to a 93 amino acid-long lysozyme fragment [34].

Our analysis of the T cell repertoire of BALB/cCr to poly-18 has characterized two clonotypes. Group B hybridomas were specific for the Glu-Tyr-Ala part of the poly-18 molecule and did not require lysine residues for activation. This clonotype was predictable from our earlier analysis using bulk T cell populations from BALB/cCr immunized *in vivo* with TNP-poly-18 [35]. Group A hybridomas, on the other hand, recognized lysine residues, and their existence was not predicted from our earlier studies. This is not surprising, however, because all of the earlier bulk T cell experiments used TNP-poly-18 as the immunizing agent, which does not activate Group A hybridomas. Analysis of all of the T cell hybridomas generated showed that they all could be placed into either Group A or Group B. These two groups represent, at the present stage of analysis, the only two clonotypes

raised by BALB/cCr mice against poly-18. The minimum-size sequence required for the activation of both groups of hybridomas is fifteen amino acids in length.

It is tempting to try to define the role of the critical residues identified in the two minimum peptide sequences required for activation of our two groups of anti-poly-18 T hybridomas. An identifiable critical residue(s) in the minimum peptide sequence could have one or more of the following roles. First, it could have a spacer role, keeping two or more other critical residues spaced at the correct distance. Secondly, it could have a conformation-stabilizing role, i.e., helix-stabilizing role, because poly-18 is α-helical under physiologic conditions [15]. Third, in the context of the trimolecular complex model, it could be involved in the antigen-IA interaction. Finally, a critical residue may be involved in the antigen-TCR interaction.

In group A hybridomas, the critical lysine at position 3 near the amino terminus of EYK(EYA)$_3$EYK may be a T cell receptor contact site for the following reasons: a) the minimum peptide length required for activation of both Group A and Group B hybridomas is the same; b) both groups of hybridomas are I-A^d-restricted, suggesting that the minimum peptides used by these groups are likely to interact with the shared restriction element through a common amino acid sequence; d) the change of only one residue of the antigen (position 3 near the amino terminus) from alanine to lysine

dictates which I-A^d-restricted clonotype gets activated; and e) TNP-poly-18 (TNP is added at the NH_2-lysine side chain) does not activate Group A hybridomas.

This suggests to us that Lys-3 in EYK(EYA)$_3$EYK is a T cell receptor contact site (epitope) in the Ia-TCR-antigen interactional complex of the Group A T cell response to poly-18. We are currently investigating the role of the other critical residues in the minimum sequence in antigen presentation to the two groups of anti-poly-18 T cell hybridomas.

TABLE 2.1

List of antigens used

Peptide	Amino Acid Sequence
Poly-18	Poly[Glu-Tyr-Lys-(Glu-Tyr-Ala)$_5$]
TNP-poly-18	Poly[Glu-Tyr-Lys(TNP)-(Glu-Tyr-Ala)$_5$]
Mann-poly-18	Poly[Glu-Tyr-Lys(Mannose)-(Glu-Tyr-Ala)$_5$]
Poly-EYA	Poly[Glu-Tyr-Ala]
Poly-EFA	Poly[Glu-Phe-Ala]
Poly-KYA	Poly[Lys-Tyr-Ala]
Poly-EYKEYA	Poly[Glu-Tyr-Lys-Glu-Tyr-Ala]
Monomer-18	Glu-Tyr-Lys-(Glu-Tyr-Ala)$_5$
Dimer-18	Glu-Tyr-Lys-(Glu-Tyr-Ala)$_5$-Glu-Tyr-Lys-(Glu-Tyr-Ala)$_5$
18-QIK	Glu-Tyr-Lys-(Glu-Tyr-Ala)$_5$-Gln-Ile-Lys
EYK(EYA)$_5$EYK	Glu-Tyr-Lys-(Glu-Tyr-Ala)$_5$-Glu-Tyr-Lys
EYK(EYA)$_4$EYK	Glu-Tyr-Lys-(Glu-Tyr-Ala)$_4$-Glu-Tyr-Lys
EYK(EYA)$_3$EYK	Glu-Tyr-Lys-(Glu-Tyr-Ala)$_3$-Glu-Tyr-Lys
EYK(EYA)$_2$EYK	Glu-Tyr-Lys-(Glu-Tyr-Ala)$_2$-Glu-Tyr-Lys
(EYA)$_4$EYK	(Glu-Tyr-Ala)$_4$-Glu-Tyr-Lys
(EYA)$_4$	(Glu-Tyr-Ala)$_4$

TABLE 2.2

Antigen specificity of poly-18 T cell line

Antigen[a]	Concentration	Stimulation Index[b] Days After Starting Culture		
		4	58	75
Poly-18	0.1 mg/ml	2.6	8.7	25
PPD	0.1 mg/ml	3.4	0.2	ND
BSA	0.1 mg/ml	ND[c]	0.7	ND
KLH	0.1 mg/ml	ND	0.8	ND

[a] T cells (10^5) were incubated with antigen and irradiated (4000 rad) BALB/cCr spleen cells (1×10^6), as described in *Materials and Methods*, in 0.2 ml of medium in 96-well microtiter plates.

[b] Stimulation index was measured by the incorporation of [^{3}H]thymidine and is expressed as: cpm in experimental group/cpm in control (no antigen).

[c] ND, not done.

TABLE 2.3

Specificity of poly-18 hybridomas

Clone No.	IL 2 Units Produced[a]				
	None	Poly-18	Poly-EYA	Mann-poly-18	TNP-poly-18
1	<10	520	<10	ND[b]	ND
2	<10	690	<10	<10	<10
3	<10	300	<10	ND	ND
4	<10	150	<10	ND	ND
5	<10	740	<10	ND	ND
6	<10	980	<10	<10	<10
7	<10	310	<10	ND	ND
8	<10	890	<10	<10	<10
9	<10	290	210	ND	ND
10	<10	240	160	ND	ND
11	<10	210	150	ND	ND
12	<10	200	170	ND	ND
13	<10	120	120	ND	ND
14	<10	140	180	ND	ND
15	<10	200	160	ND	ND
16	<10	150	160	ND	ND

[a] T cell hybrids (10^5) were incubated with 10^6 irradiated (4000 rad) BALB/cCr spleen cells for 48 h. Antigens were added at a concentration of 0.1 mg/mL. IL 2 release was assayed in a secondary culture as described [22]. IL 2 units were calculated by the logit transformation method of Hedrick et al. [23].

[b] ND, not done.

TABLE 2.4

Fine specificity of Group A hybridomas

Antigen	IL 2 Units Produced				
	A1.1	A2.1	A3.1	A4.1	A5.1
None	<10	<10	<10	<10	<10
Poly-18	800	1110	1020	580	930
Poly-KYA	<10	<10	<10	<10	<10
Poly-EYKEYA	<10	<10	<10	<10	<10
Monomer-18	<10	<10	<10	<10	<10
Dimer-18	610	1070	720	330	860
18-QIK	400	500	830	290	790
$EYK(EYA)_5EYK$	730	500	630	270	720
$EYK(EYA)_4EYK$	450	450	670	240	560
$EYK(EYA)_3EYK$	610	380	630	210	810
$EYK(EYA)_2EYK$	<10	<10	<10	<10	<10
$(EYA)_4EYK$	<10	<10	<10	<10	<10

T cell hybrids (10^5) were incubated with 10^6 irradiated (4000 rad) BALB/cCr spleen cells for 48 h. Antigens were added at a concentration of 0.1 mg/mL. IL 2 release was assayed in a secondary culture as described [22]. IL 2 units were calculated by the logit transformation method of Hedrick et al. [23].

TABLE 2.5

Fine specificty of Group B hybridomas

Antigens	IL 2 Units Produced[a] by Cloned Hybridomas				
	B1.1	B2.1	B3.1	B4.1	B5.1
None	<10	<10	<10	<10	<10
Poly-18	670	880	780	680	1460
Poly-EYA	770	1120	710	960	880
Poly-EFA	160	260	150	210	220
Poly-KYA	<10	<10	<10	<10	<10
Monomer-18	600	800	700	600	1000
$EYK(EYA)_5EYK$	340	220	330	250	840
$EYK(EYA)_4EYK$	100	150	140	100	160
$EYK(EYA)_3EYK$	<10	<10	<10	<10	<10
$EYK(EYA)_2EYK$	<10	<10	<10	<10	<10
$(EYA)_4$	<10	<10	<10	<10	<10
$(EYA)_4EYK$	150	90	110	70	210

[a] T cell hybrids (10^5) were incubated with 10^6 irradiated (4000 rad) BALB/cCr spleen cells for 48 h. Antigens were added at a concentration of 0.1 mg/mL. IL 2 release was assayed in a secondary culture as described [22]. IL 2 units were calculated by the logit transformation method of Hedrick et al. [23].

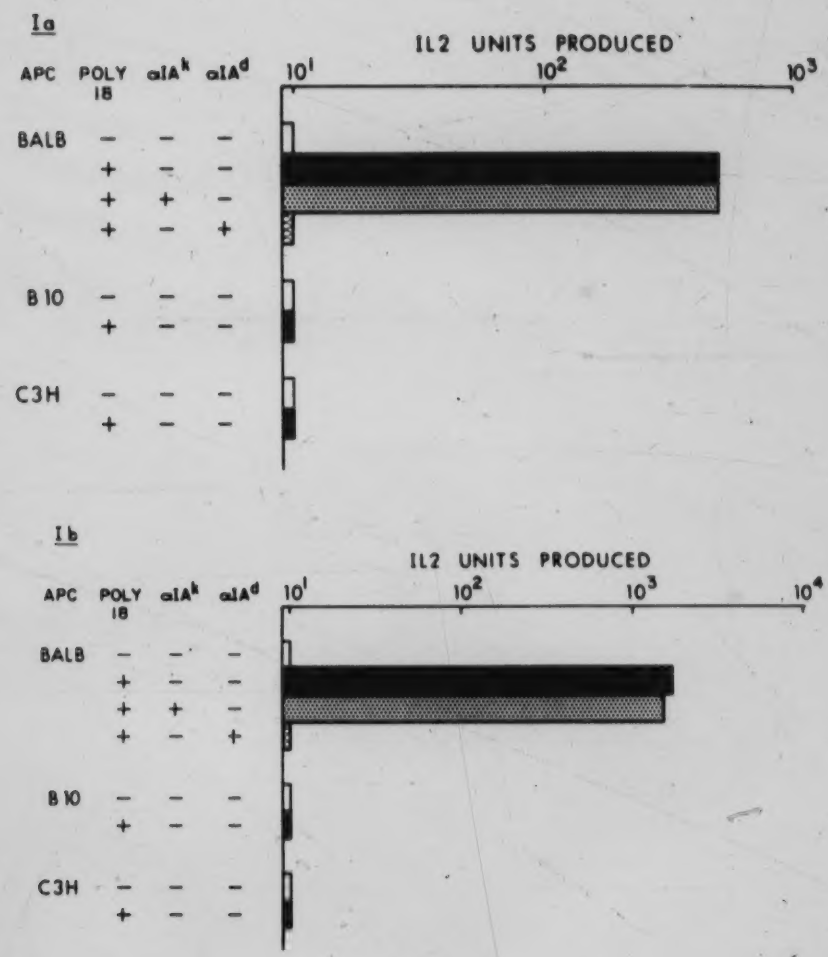

Figure 2.1. T cell hybridomas A.1.1 (*A*) and B.1.1 (*B*) were stimulated with 10^6 irradiated (4000 rad) spleen cells and 100 μg/mL poly-18. Anti-IA^k (10.3.6) and anti-IA^d (MKD6) were added at a 1/20 dilution. APC used were from BALB/cCr (H-2^d), C57BL/10J (H-2^b), or C3H.HeJ (H-2^k) strains.

REFERENCES

1. **Barcinski, M.A., and A.S. Rosenthal.** 1977. Immune response gene control of determinant selection. I. Intramolecular mapping of the immunogenic sites on insulin recognized by guinea pig T and B cells. *J.Exp.Med. 145*:726.

2. **Corradin, G., and J.M. Chiller.** 1979. Lymphocyte specificity to protein antigens. II. Fine specificity of T cell activation with cytochrome c and derived peptides as antigenic probes. *J.Exp.Med. 149*:436.

3. **Infante, A.J., M.Z. Atassi, and C.G. Fathman.** 1981. T cell clones reactive with sperm whale myoglobin. Isolation of clones with specificity for individual determinants on myoglobin. *J.Exp.Med. 154*:1342.

4. **Maizels, R.M., J.A. Clarke, M.A. Harvey, A. Miller, and E.E. Sercarz.** 1980. Epitope specificity of the T cell proliferative response to lysozyme: proliferative T cells react predominantly to different determinants from those recognized by B cells. *Eur.J.Immunol. 10*:509.

5. **Thomas, D.W., K.H. Hsieh, J.L. Schauster, M.S. Mudd, and G.D. Wilner.** 1980. Nature of T lymphocyte recognition of macrophage associated antigens. V. Contribution of individual peptide residues of human fibrinopeptide B to T lymphocyte responses. *J.Exp.Med. 152*:620.

6. **Michaelides, M.M., M.S. Sandrin, G.M. Morgan, I.F.C. McKenzie, R. Ashman, and R.W. Melvold.** 1981. Ir gene function in an I-A subregion mutant B6.C H-2bm12. *J.Exp.Med. 153*:464.

7. **Lin, C.C.S., A.S. Rosenthal, H.C. Passmore, and T.H. Hanson.** 1981. Selective loss of an antigen specific Ir gene function in an I-A mutant B6.C H-2bm12 is an antigen presenting cell defect. *Proc.Natl.Acad.Sci.USA 78*:6406.

8. **Pierce, S.K., N.R. Klinman, P.H. Maurer, and C.F. Merryman.** 1980. Role of the major histocompatibility gene products in regulating the antibody response to dinitrophenylated poly(L-Glu56-L-Lys35-L-Phe9). *J.Exp.Med. 152*:336.

9. **Singer, A., H.B. Dickler, and R.J. Hodes.** 1977. Cellular and genetic control of antibody responses in vitro. II. Ir gene control of primary IgM responses to trinitrophenyl conjugates of Poly L-(Tyr,Glu)-Poly D,L-Ala-Poly L-Lys and Poly L-(His,Glu)-Poly D,L-Ala-Poly L-Lys. *J.Exp.Med. 146*:1096.

10. **Keck, K.** 1975. Ir gene control of carrier recognition. I. Immunogenicity of bovine insulin derivatives. *Eur.J.Immunol. 5*:801.

11. **Rossenwasser, L.J., M.A., Barcinski, R.H., Schwartz, and A.S. Rosenthal.** 1979. Immune response gene control of determinant selection. II. Genetic control of the murine T lymphocyte proliferative response to insulin. *J.Immunol. 123*:471.

12. **Hill, S., and E.E. Sercarz.** 1975. Fine specificity of an immune response gene for the gallinaceous lysozymes. *Eur.J.Immunol. 5*:317.

13. **Solinger, A.M., M.E. Ultee, E. Margoliash, and R.H. Schwartz.** 1979. The T lymphocyte proliferative response to cytochrome c. I. Demonstration of a T cell heteroclitic proliferative response and identification of a single topographic antigenic determinant on pigeon cytochrome c whose immune recognition requires two complementing major histocompatibility complex linked response genes. *J.Exp.Med. 150*:830.

14. **Goodman, J.W., and E.E. Sercarz.** 1983. The complexity of structures involved in T cell activation. *Ann.Rev.Immunol. 1*:465.

15. **Barton, M., B. Singh, and E. Fraga.** 1977. Synthetic polypeptide antigens of defined geometry. *J.Am.Chem.Soc. 99*:8491-8498.

16. **McDevitt, H.O., and M. Sela.** 1965. Genetic control of the antibody response. I. Demonstration of determinant specific differences in response to synthetic polypeptide antigens in two strains of inbred mice. *J.Exp.Med. 122*:517.

17. **Schwartz, R.H., M.E. Dorf, B. Benacerraf, and W.E. Paul.** 1976. The requirement for two complementing Ir-GLphi

immune response genes in the T-lymphocyte proliferative response to poly-(Glu53-Lys36-Phe11). *J.Exp.Med. 143*:897.

18. **Lee, K.-C., B. Singh, M.A. Barton, A. Procyshyn, and M. Wong.** 1979. A simple reliable system for studying antigen-specific murine T cell proliferation. *J.Immunol.Methods 25*:159.

19. **Kimoto, M., and C.G. Fathman.** 1980. Antigen-reactive T cell clones. I. Transcomplementing hybrid I-A-region gene products function effectively in antigen presentation. *J.Exp.Med. 152*:759.

20. **Kappler, J.W., B. Skidmore, J. White, and P. Marrack.** 1981. Antigen-inducible, H-2 restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and H-2 recognition. *J.Exp.Med. 153*:1198-1214.

21. **Nabel, G., M. Fresno, A. Chessman, and H. Cantor.** 1981. Use of cloned populations of mouse lymphocytes to analyze cellular differentiation. *Cell 23*:19.

22. **Heber-Katz, E., R.H. Schwartz, L.A. Matis, C. Hannum, T. Fairwell, E. Appella, and D. Hansburg.** 1982. The contribution of antigen presenting cell major histocompatibility complex gene products to the specificity of antigen induced T cell activation. *J.Exp.Med. 155*:1086-1099.

23. **Hedrick, S.M., L.A. Matis, T.T. Hecht, L.E. Samelson, D.L. Longo, E. Heber-Katz, and R.H. Schwartz.** 1982. The fine specificity of antigen and Ia determinant recognition by T cell hybridoma clones specific for pigeon cytochrome c. *Cell 30*:141-152.

24. **Oi, V.T., P.P. Jones, J.W. Goding, and L.A. Herzenberg.** 1978. Properties of monoclonal antibodies to mouse Ig allotypes, H-2 and Ia antigens. *Curr.Top.Microbiol.Immunol. 81*:115.

25. **Araneo, B.A., and J.A. Kapp.** 1980. H-2 linked Ir gene control of T cell proliferative responses to the synthetic terpolymer L-glutamic acid60-L-alanine30-L-tyrosine10 (GAT). I. Requirement for T cell activation in responder and non-responder mice. *J.Immunol. 124*:1492.

26. **Kimoto, M., T.J. Krenz, and C.G. Fathman.** 1981. Antigen reactive T cell clones. III. Low responder antigen presenting cells function effectively to present antigen to selected T cell clones derived from (high responder x low responder) F1 mice. *J.Exp.Med. 154*:883.

27. **Ishii, N., C.N. Baxevanis, Z.A. Nagy, and J.J. Klein.** 1981. Responder T cells depleted of alloreactive cells to antigen presented on allogeneic macrophages from nonresponder strains. *J.Exp.Med. 154*:978.

28. **Clark, R.M., and E.M. Shevach.** 1981. Generation of T cell colonies from responder strain 2 guinea pigs that recognize the copolymer L-glutamic acid, L-lysine in association with nonresponder strain 13 Ia antigens. *J.Exp.Med. 155*:635.

29. **Rosenthal, A.S.** 1978. Determinant selection and macrophage function in genetic control of the immune response. *Immunol.Rev. 40*:135.

30. **Rosenthal, A.S., M.A. Barcinski, and J.T. Blake.** 1977. Determinant selection is a macrophage mediated immune response gene function. *Nature 267*:156.

31. **Hansburg, D., C. Hannum, J.K. Inman, E. Appella, E. Margoliash, and R.H. Schwartz.** 1981. Parallel cross-reactive patterns of two sets of antigenically distinct cytochrome c peptides: possible evidence for a presentational model of Ir gene function. *J.Immunol. 127*:1844.

32. **Heber-Katz, E., D. Hansburg, and R.H. Schwartz.** 1983. The Ia molecule of the antigen-presenting cell plays a critical role in immune response gene regulation of T cell activation. *J.Mol.Cell.Immunol. 1*:3-14.

33. **Hansburg, D., E. Heber-Katz, T. Fairwell, and E. Appella.** 1983. Major histocompatibility complex-controlled, antigen-presenting cell-expressed specificity of T cell antigen recognition. Identification of a site of interaction and its relationship to Ir genes. *J.Exp.Med. 158*:25-39.

34. **Manca, F., J.A. Clarke, A. Miller, E.E. Sercarz, and N. Shashtri.** 1984. A limited region within hen egg

white lysozyme serves as the focus for diversity of T cell clones. *J.Immunol. 133*:2075.

35. **Singh, B., K.-C. Lee, E. Fraga, A. Wilkinson, M. Wong, and M. Barton.** 1980. Minimum peptide sequences necessary for priming and triggering of humoral and cell mediated immune responses in mice: use of synthetic peptide antigens of defined structure. *J.Immunol. 124*:1336-1343.

CHAPTER 3

Contribution of antigen processing to the recognition of synthetic peptide antigens by T cell hybridomas A.1.1 and B.1.1[1]

Antigen mediated activation of helper T cells requires the participation of accessory cells, which perform several functions in this process (reviewed in [1,2]). Among these, the primary function is the presentation of antigen to the T cells in conjunction with cell surface proteins encoded by the I region of the major histocompatibility complex. A large body of evidence suggests that antigen and Ia are both recognized by the same receptor on T cells [3]. Furthermore, there is functional evidence that antigen and Ia must interact to induce T cell stimulation [4], as well as direct evidence that Ia binds antigen [5,6]. These findings strongly support a model of T cell activation requiring the formation of a ternary complex between the T cell receptor, antigen and Ia [7]. On the other hand, antigen presentation is preceded, in many cases, by an antigen handling step which is referred to as processing [8]. This step involves uptake of antigen by the accessory cell, its partial degradation or

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denaturation, presumably in endosomes [9], and its re-expression on the accessory cell surface where interaction with the T cell occurs. This antigen processing event apparently serves to isolate or expose immunogenic portions of antigen and to make them accessible to T cell recognition on the antigen presenting cell (APC) surface.

Antigen processing has been shown to be necessary for T cell activation in many systems [8,10-14] especially with particulate antigens such as bacteria but also with most large polypeptides or proteins. The capacity of the latter antigens to stimulate specific T cells can be abrogated by experimentally blocking the processing function of APC's and can subsequently be restored by denaturation [15,16] or fragmentation [14] of the antigens prior to their addition to culture. In this respect, Shimonkevitz et al. have shown that fragmented ovalbumin could activate ovalbumin-specific T cell hybridomas when presented by APC's rendered unable to process antigen by glutaraldehyde fixation [14]. This was subsequently confirmed by Watts et al. using a planar membrane system [17].

Antigen size, however, is not the only factor which determines whether it requires processing to stimulate T cells. Buus and Werdelin have provided evidence that T cells unresponsive to a 10 amino acid peptide from the angiotensin lineage can be triggered by this antigen if a single amino acid is removed from the peptide [18]. This suggests that the function of processing is not only to reduce the antigen

to a manageable size for interaction with the T cell, but also to present it in an immunogenic form. Further evidence for this comes from studies of the processing requirements of a cytochrome C reactive T cell clone [19], where it appears that proper antigen-T cell interaction requires prior disruption of an internal bond between 2 residues, Glu 61 and Lys 99, of the cytochrome C molecule. A myoglobin reactive T cell clone has also been shown to require an antigen processing step which appears to involve unmasking of certain regions of the molecule normally buried in the native antigen [16].

The antigen processing capacity of accessory cells can be shut off by drugs such as chloroquine [8,20] or fixation of the cell surface with cross-linking reagents such as glutaraldehyde [14]. These treatments have been used to identify critical epitopes [19] on antigens for T cell activation and have provided insights into the nature of the immunogenic moieties of antigen which govern T cell responses.

We reported earlier on the fine specificity of poly 18 reactive I-A^d restricted T cell hybridomas generated in this laboratory [21]. Poly 18, poly EYK(EYA)$_5$, is an α-helical polypeptide antigen of defined sequence [22-24]. A panel of minimum peptides and their analogs were used for these studies which revealed two groups of hybridomas, group A exemplified by clone A.1.1., and group B represented by

B.1.1. Group A hybridomas recognize EYK(EYA)$_4$ and group B hybridomas are activated by (EYA)$_5$.

In this report, we examine whether there is a requirement for antigen processing in the activation of poly 18 reactive hybridomas A.1.1 and B.1.1, using TA3, an I-$A^{d/k}$/I-$E^{d/k}$ bearing B cell hybridoma [25] which presents antigen efficiently to T cells. We also examine the role played by specific amino acids of the antigens in activation of hybridoma B.1.1. We have found that poly 18 and various minimum immunogenic peptides related to poly 18 could be efficiently presented to both T cell hybridomas without processing. We also present evidence for an interaction between antigen and a cell surface structure of the presenting cell, which is mediated by a specific amino acid of the antigen and plays a role in T cell activation.

MATERIALS AND METHODS

Animals. BALB/cCr mice were bred at the Ellerslie Animal Farm of the University of Alberta, or were purchased from The Jackson Laboratory, Bar Harbor, ME.

Antigens. The synthetic strategy for poly 18 and the various related peptides was described previously [21,22]. In brief, it utilized a fragment condensation scheme with selectively deprotected amino and carboxyl components, using the dicyclohexylcarbodiimidehydroxybenzotriazole method. The protecting groups used were tert-butyl ester for the glutamic acid side chain group, tert-butyl ether for the tyrosine side chain group, 2-(4-biphenyl)-isopropoxycarbonyl for the amino terminus, and phenyl ester for the carboxyl terminus. Protected peptides were purified by Sephadex LH-20 gel filtration in DMF. Amino acid analysis and thin-layer chromatography were performed at every stage of coupling to monitor the completion of reactions. Final deprotections were performed with HBr in TFA as described [22]. Peptides and polypeptides were purified by HPLC. Samples were dissolved in water at pH 7.2 (10% NH_4OH) and applied to a C-18 reversed phase semipreparative Synchropak RP-P column. Peptides and polypeptides were eluted with a linear gradient from water to acetonitrile (1.37% acetonitrile per minute). Fractions were pooled and lyophilized. Amino acid analyses

were performed on the purified peptides by using a Beckman System 6300 amino acid analyzer. For this purpose samples were hydrolysed in 6N HCl containing 0.1% phenol in sealed evacuated tubes for 20 h at 110°C. All peptides gave acceptable ratios of the various amino acids. For functional assays, peptides were dissolved in saline by adjusting pH to 7.2 with 0.1N NaOH and were sterilized by filtration through a 0.22 μm filter. Table I lists the synthetic antigens used in this study.

T cell hybridomas. Poly 18-reactive, I-A^d-restricted T cell hybridomas [21] and a beef insulin-reactive, I-A^d-restricted T cell hybridoma [26,27] were generated in this laboratory and have been previously described.

Antigen presenting cells. For antigen presentation, irradiated spleen cells (4000 rads) or cells of the I-$A^{d/k}$/I-$E^{d/k}$ bearing B cell hybridoma TA3 [25] were added at 10^6 or 10^4 cells per well respectively. For glutaraldehyde fixation, cells were washed twice with Hepes buffered balanced salt solution (HBBSS) and 5 x 10^6 to 10^7 cells/mL in HBBSS were treated with a final concentration of 0.05% of glutaraldehyde (Fisher Scientific), as described [14]. After half a minute at room temperature, fixation was stopped by addition of an equal volume of 0.2 M lysine (Sigma) in HBBSS. Cells were washed twice in HBBSS before use. Efficacy of the glutaraldehyde treatment was determined by [^{3}H]-leucine uptake by the fixed cells, following the protocol of Shimonkevitz et al. [14]. Maximum uptake by fixed cells was

3% of normal cell uptake. For chloroquine treatment, irradiated cells were washed twice in RPMI, suspended at 5 x 10^6 cells/mL in 0.8 mM Chloroquine in RPMI and incubated for 40 min at 37°C on a roller drum. Cells were washed twice in RPMI before use.

IL-2 assay. T hybridoma cells (5 x 10^4 or 10^5) were co-cultured with 10^6 irradiated (4000 rad) spleen cells or 10^4 TA3's, treated or untreated, in the presence or absence of antigen in 200 µL of culture medium (RPMI 1640 containing 10 mM HEPES, 2 mM glutamine, 5 x 10^{-5} M 2-mercaptoethanol, 10% heat inactivated fetal calf serum, and 1 U/mL penicillin-streptomycin (Gibco). After 24 to 42 hours, supernatants were collected and were assayed for IL-2 content in a secondary culture by using thymocytes [28] (Figure 1 and Table 2) or the IL-2 dependent line HT-2 (originally described by Dr. J. Watson [29], and provided by Dr. R.H. Schwartz, NIH, Bethesda, MD). Cells (10^4 HT-2's or 10^5 thymocytes) were cultured for 48 h (HT-2) or 4 days (thymocytes) with 4 serial dilutions of primary supernatant (starting at 25% primary supernatant), and the degree of stimulation was measured by the incorporation of [^{3}H]-thymidine (40 kBq/well). IL-2 units were calculated according to the method of Hedrick et al. [30], using a computer program devised and kindly provided to us by B.S. Davis (Immunex Corp., Seattle, WA). IL-2 units in the absence of antigen or APC were less than 10.

RESULTS

Antigen processing is not required for activation of poly 18 specific T cell hybridomas. Cells of the B hybridoma TA3 were fixed with glutaraldehyde and used to present poly 18 to A.1.1 and B.1.1 T hybridomas. As shown in Table 3.2 and Figure 3.1A, it was found that both hybridomas could be activated by poly 18 presented on glutaraldehyde-treated APC's. However, there was a noticeable reduction in the magnitude of the response at comparable antigen doses presumably because the glutaraldehyde treatment modifies cell surface structures on the APC which are necessary for optimal T cell-APC-antigen interactions. Although treatment of the antigen presenting cells with glutaraldehyde affects the T cell response, the results shown here suggest that processing of poly 18 is not a requirement for the activation of hybridomas A.1.1 and B.1.1.

To ascertain that the glutaraldehyde fixation protocol we used effectively blocked processing, the beef insulin specific T hybridoma A20.2.15 was tested with fixed APC. As Figure 3.2 shows, glutaraldehyde treated TA3's or splenocytes which were exposed to antigen prior to fixation could present beef insulin to A20.2.15 cells. In contrast, APC's fixed prior to being exposed to antigen were unable to stimulate this hybridoma.

Presentation of peptide antigens to hybridoma A.1.1 with fixed TA3's. We reported earlier on the use of synthetic peptides related to poly 18 to investigate the antigen fine specificity and minimum size requirements for the triggering of both groups of T hybridomas [21] (Table 3.1). To determine if antigen processing was a requirement for the activation of group A hybridoma A.1.1 by poly 18 analogs and the minimum peptide, the T hybrid was cultured with various antigens and glutaraldehyde fixed TA3's. Table 3.2 shows that synthetic antigens which activate this group when presented by normal TA3's can also activate when presented on fixed cells. However, consistent with previous results obtained using poly 18, the magnitude of the response generated with glutaraldehyde fixed TA3's was lower than that resulting from stimulation with untreated cells.

The minimum sequence that triggers hybridoma B.1.1 is A(EYA)$_4$. Previous studies in this laboratory established that group B hybridomas could be stimulated by (EYA)$_4$EYK/A [21]. In order to determine the role of the N-terminal residues, Glu and Tyr, the following peptide, A(EYA)$_4$ was synthesized and tested for its ability to elicit a response from hybridoma B.1.1. As shown in Table 3.3, A(EYA)$_4$ was stimulatory and was found to be the minimum sequence able to stimulate this hybridoma, since (EYA)$_4$ could not activate it [21].

Lysine residues in the antigen interfere with optimal presentation to hybridoma B.1.1 by glutaraldehyde fixed TA3's. For this hybridoma, initial results at low antigen doses suggested that certain antigens might require processing to be stimulatory. However at higher antigen concentrations, all poly 18 related peptides and polypeptides tested could activate this hybridoma as shown in Figure 3.1. Nevertheless it was apparent that the effect of glutaraldehyde fixation of APC's was not the same for all antigens used. A pattern of reactivity has emerged, which was consistent over six experiments performed. Figure 3.1A shows that the T hybridoma response to poly 18 and to poly EYA presented by normal TA3's was approximately the same over most of the dose range used. However, responses with glutaraldehyde fixed TA3's were reduced almost 10-fold in the case of poly 18 whereas a 2 to 3 fold drop was seen with poly EYA. A similar, although less pronounced difference is shown in Figure 3.1B between EYK$(EYA)_5$ and A$(EYA)_4$: when glutaraldehyde fixed APC's were used, a 10 fold decrease in response was observed with EYK$(EYA)_5$ compared to a 4 to 5 fold decrease with A$(EYA)_4$. These differences in responsiveness correlated with the presence or absence of lysine residues in the antigens. That is, peptides or polypeptides containing lysine residues were found to suffer a larger decrease in potency than antigens with no lysine residues, when presented on glutaraldehyde fixed TA3's.

Increasing antigen doses up to 0.25 mg/mL did not overcome this effect.

Lysine residues of antigen do not interfere with presentation to hybridoma B.1.1 by chloroquine treated splenocytes. In contrast to results obtained with glutaraldehyde fixed TA3's, the presenting ability of chloroquine treated APC's was not linked to the presence of lysine in the antigens. That is, the T cell responses to both poly 18 and poly EYA were affected to the same extent when chloroquine treated APC's were used (Figure 3.3A). Similarly, peptides $(EYA)_5$ and $(EYA)_4EYK$ were presented equally well by treated or untreated spleen cells (Figure 3.3B). To rule out the possibility that the chloroquine treatment might be allowing some processing to occur, the beef insulin specific T hybridoma A20.2.15 was given antigen on treated APC. As shown in Figure 3.4, A20.2.15 failed to respond to beef insulin presented by chloroquine treated spleen cells, confirming the effectiveness of this protocol to inhibit antigen processing by accessory cells.

DISCUSSION

The processing requirements of two T cell hybridomas with distinct fine specificities for the synthetic polypeptide, poly 18, were examined. Two major findings emerged from this study. First, poly 18 could be presented to both hybridomas by glutaraldehyde fixed or chloroquine treated antigen presenting cells. This constitutes strong evidence that antigen processing is not required for activation of poly 18 specific T cell hybridomas. Both glutaraldehyde and chloroquine have been shown to be potent inhibitors of processing in a number of systems [8,12-16,19].

Glutaraldehyde inhibits the cell's metabolic processes, presumably by cross-linking surface molecules and thereby preventing cellular exchange with the environment. Chloroquine [20] is thought to act by raising the intraendosomal or intralysosomal pH, and in this way, interferes with proteolysis and/or denaturation of antigen. In this study, chloroquine treatments were only performed on splenocytes and not on TA3's, as there is some doubt concerning the efficacy of the drug to prevent processing by B cell hybridomas over the period of time required to achieve T cell stimulation [19].

The results presented here suggest that antigen fragmentation is not required for the activation of T cell

hybridomas A.1.1. and B.1.1. Similar results have been obtained using leupeptin as an inhibitor of processing (unpublished observation). On the other hand, processing of beef insulin is required for presentation to a beef insulin specific T cell hybridoma under similar conditions.

It has been proposed [31-33] that one of the common properties of antigenic sites for T cell activation is their capacity to form amphipathic structures. These can be of two types: they can either be determined by periodicity in hydrophobic residues or by sequential changes in hydrophobicity. Poly 18, with its repeating sequence, is a candidate for the first type. In fact, circular dichroism studies of poly 18 in our laboratory have indicated a partially helical conformation in solution under physiological conditions [22]. Since the helical content of poly 18 would be expected to increase in the more hydrophobic environment created at the T cell-APC interface, this may allow proper interaction of poly 18 with Ia without any additional requirement for unfolding or degradation of the antigen.

However, upon closer examination, if poly 18 is envisioned as forming an alpha-helix (Figure 3.5), it is not immediately obvious how this conformation generates an amphipathic structure with the necessary hydrophobic face. The amino acid residues most likely to make up the hydrophobic face, alanines, do not cluster to one side of the helix. Instead, they are found all around it. Moreover,

they are in close proximity to both a tyrosine and a glutamate, and to a lysine every fifth turn of the helix. On the other hand, it is clear (Figure 3.6) that, in an alpha-helical conformation, alanine residues would line up to form a hydrophobic ridge spiraling up the outside of the molecule. The presence of such ridges, and of the corresponding grooves between them, is believed to play a role in protein-protein interactions [34,35]. In the case of poly 18, this might be sufficient to generate the appropriate interaction site postulated by the DeLisi-Berzofsky model. In addition, tyrosine residues which line up to form a ridge parallel to the alanine ridge may, in the case of poly 18, be involved in hydrogen bonding with the glutamates and, in this state, give rise to a structure that would be more compatible with hydrophobic interactions. Evidence for hydrogen bonds between the side chains of Tyr and Glu residues in poly YAE, a molecule very similar to Poly 18 has been presented by Ramachandran et al. [36]. Figure 3.6 shows how these hydrogen bonds may draw the charged and polar side chains away from the alanine ridge.

Consistent with the idea that alanine residues may form a hydrophobic ridge and that this feature may be required for appropriate interaction between antigen, T cell and APC is the observation that poly EYKEYA does not stimulate hybridomas A.1.1 or B.1.1 [21]. When this polypeptide is modeled in an alpha-helical conformation, no hydrophobic ridge can be generated. Instead, alanine residues alternate

with lysine residues along the ridge which corresponds most closely to the alanine ridge of poly 18.

If, alternatively, the conformation of poly 18 approaches that of a 3_{10} helix, then the alanine residues cluster together and generate a hydrophobic face which conforms more strictly to the amphipathic model.

The second major finding of this study is that the presence of lysine residues on antigens correlates with a substantial decrease in activation of hybridoma B.1.1 when glutaraldehyde fixed TA3's act as antigen presenting cells. Two interpretations can be invoked to explain these results. In the first scenario, the potency of lysine containing antigens is low for this hybridoma but can be increased by a processing event which converts them to more immunogenic molecules with lysine removed or altered. In the second case, glutaraldehyde modifies an APC surface molecule (presumably Ia) in a way that greatly impedes the interaction between this molecule, the TCR and antigens containing lysine. If the observed decrease in response with antigens that have lysine is due to a processing requirement for these antigens, one would expect that any treatment of the APC's which inhibits processing should give similar results. But in fact, when antigen presenting cells are treated with chloroquine, the T hybridoma responses are only slightly affected and this independently of whether antigens contain lysine or not. This suggests that the effect seen with glutaraldehyde fixed APC's is not due to a processing

requirement but is the result of the glutaraldehyde treatment itself. A possible explanation is that glutaraldehyde has modified the desetope (antigen interaction site on Ia) in a way that hinders interaction with lysine containing antigens to a greater degree than with lysine free antigens. A corollary of this is that lysine residues appear to interact with the APC surface and may be contact sites of antigen with Ia. Such interactions of antigen with Ia have been shown to occur [4,7,13] and there is direct evidence for binding of antigen and Ia [5,6].

An alternative explanation is that some site on the APC surface, not related to the Ia site required for T cell activation, has been modified by glutaraldehyde and, as a result, displays a higher avidity for lysine-bearing antigens. In this scenario, an irrelevant site on the APC, perhaps on Ia, competes for antigen with the Ia site that is involved in B.1.1 activation.

At this point, it is not possible to distinguish between these possibilities. In any case, the evidence presented here suggests that certain residues of antigen can interfere, directly or indirectly, with productive antigen-Ia interactions required for T cell activation. Similar events may be involved in some Ir gene defects, where the amino acids of the antigen do not allow productive interactions with Ia antigens either because they directly hinder interaction with the relevant sites for T cell activation or preferentially interact with irrelevant sites on Ia or on the

APC surface. Such mechanisms may help explain why mice of the H-2^b haplotype are responders to poly EYA, but unable to respond to poly 18.

TABLE 3.1

Reactivity pattern of Poly 18 specific T cell hybridomas with the antigens used in this study

Antigens[a]	Response of T cell hybridoma[b]	
	A.1.1	B.1.1
Poly 18 [Poly EYK$(EYA)_5$]	+	+
Poly EYA	-	+
EYK$(EYA)_5$EYK	+	+
EYK$(EYA)_3$EYK	+	-
EYK$(EYA)_5$	-	+
$(EYA)_4$EYK	-	+
$(EYA)_5$	-	+

[a] Amino acids are designated by the single letter code: A, alanine; E, glutamic acid; K, lysine; Y, tyrosine.

[b] Responses were measured by IL-2 secretion in the presence of appropriate antigen and I-A^d containing APC as described in Materials and Methods.

TABLE 3.2

Presentation of poly 18 and synthetic peptides to hybridoma A.1.1 by glutaraldehyde fixed TA3 cells[a]

Antigen	IL-2 Units	
	Untreated TA3	Fixed TA3
None	<10	<10
Poly 18[b]	450	350
$EYK(EYA)_5EYK$	168	100
$EYK(EYA)_3EYK$	292	107

[a] T hybridoma cells were cultured with fixed or untreated TA3 cells and 100 μg/ml of antigen as described in Materials and Methods.

[b] Poly 18 is a high molecular weight fraction obtained by gel filtration on Sephadex G-50 [22] and further fractionated by high performance liquid chromatography as described in Materials and Methods.

TABLE 3.3

Minimum amino acid sequence of antigen required for activation of T cell hybridoma B.1.1[a]

Antigen	IL-2 units	
	Expt I	Expt II
None	<10	<10
Poly 18	400	ND[b]
Poly EYA	450	700
A(EYA)4	250	200
(EYA)4	<10	<10

[a] T hybridoma cells were cultured with TA3 cells and 100 µg/mL of various antigens as described in Materials and Methods.

[b] Not determined.

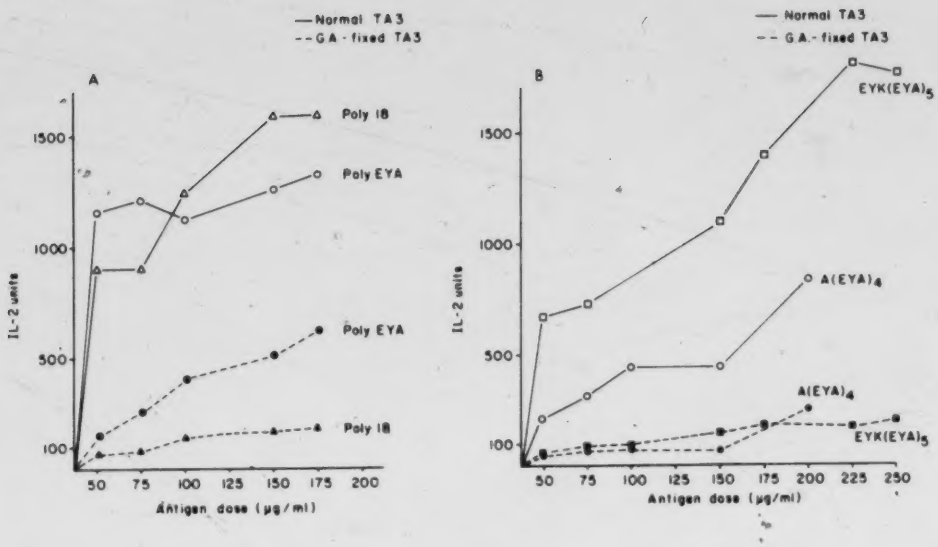

Figure 3.1. Effect of glutaraldehyde fixation of APC on presentation of poly 18 and poly EYA (A), and synthetic peptides $EYK(EYA)_5$ and $A(EYA)_4$ (B) to clone B.1.1. Poly 18 and poly EYA are high molecular weight fractions obtained by gel filtration on Sephadex G-50 [22] and further fractionated by high performance liquid chromatography as described in Materials and Methods.

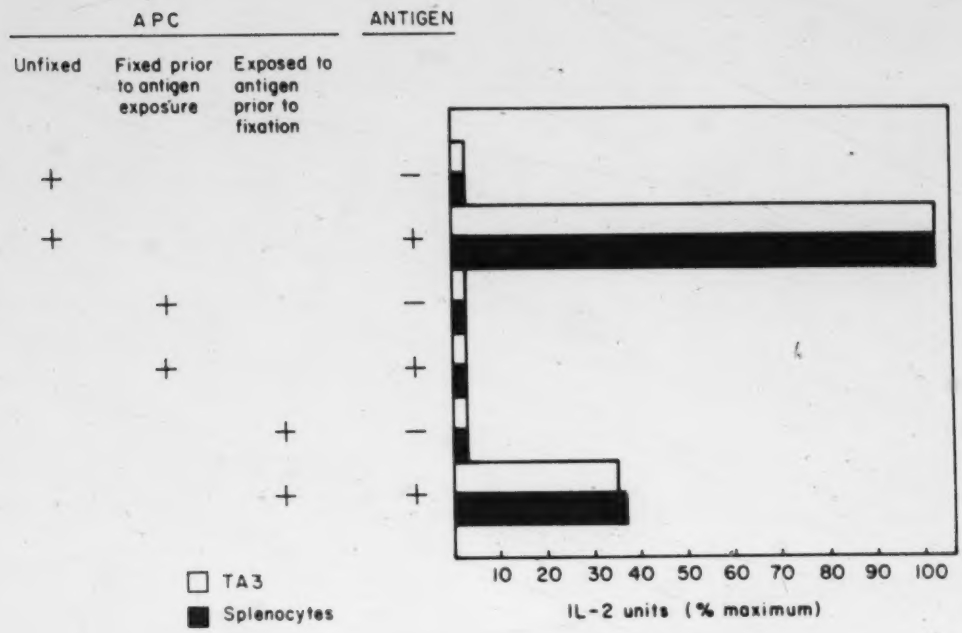

Figure 3.2. Antigen presentation to beef insulin specific T hybridoma A20.2.15 by TA3's (hatched bars) or spleen cells (solid bars) fixed with glutaraldehyde before or after antigen exposure. The antigen, beef insulin, was used at 100 μg/mL. Results are expressed as percent maximal response in IL-2 units.

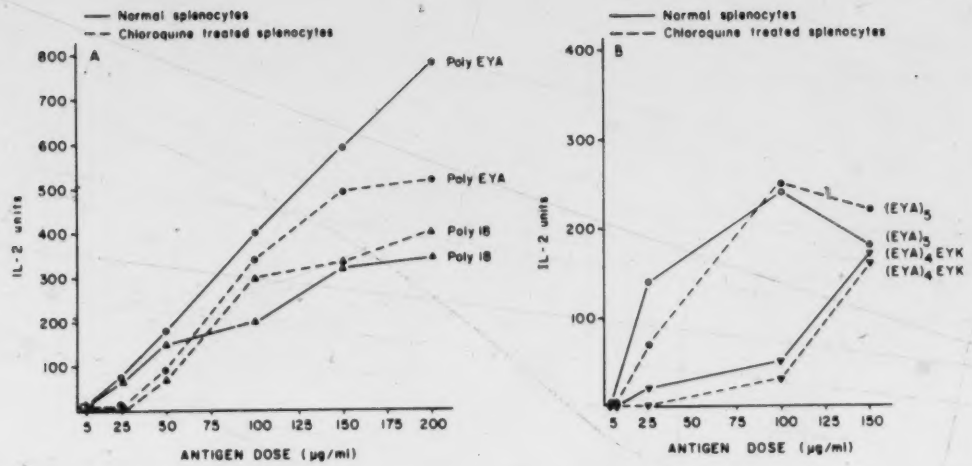

Figure 3.3. Antigen presentation to clone B.1.1 by chloroquine treated splenocytes. Antigens used are poly 18 and poly EYA (A), and $(EYA)_5$ and $(EYA)_4EYK$ (B). IL-2 units in the absence of antigen: <10. Poly 18 and poly EYA are high molecular weight fractions obtained by gel filtration on Sephadex G-50 [22] and further fractionated by high performance liquid chromatography as described in Materials and Methods.

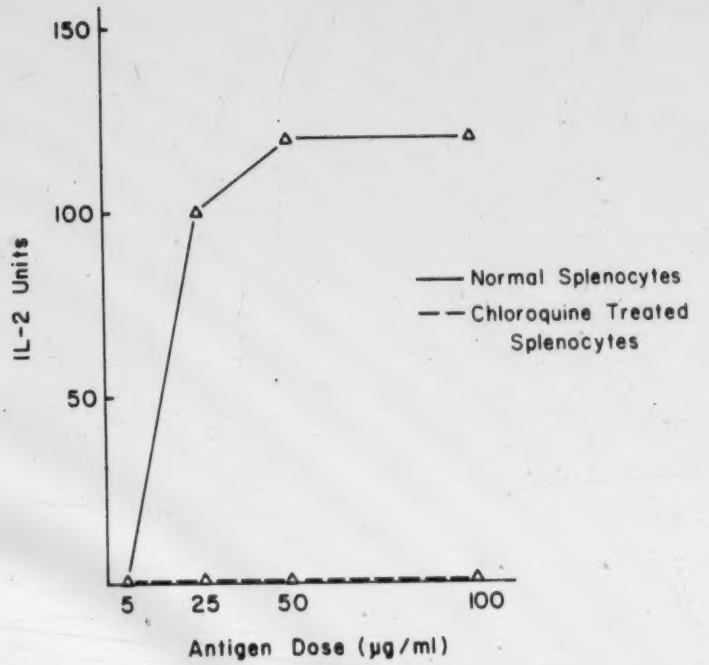

Figure 3.4. Antigen presentation to beef insulin specific T hybridoma A20.2.15 (10^5 cells/well) by chloroquine treated splenocytes (10^6 cells/well). Results are expressed in IL-2 units as described in Materials and Methods. IL-2 units in the absence of antigen were less than 10.

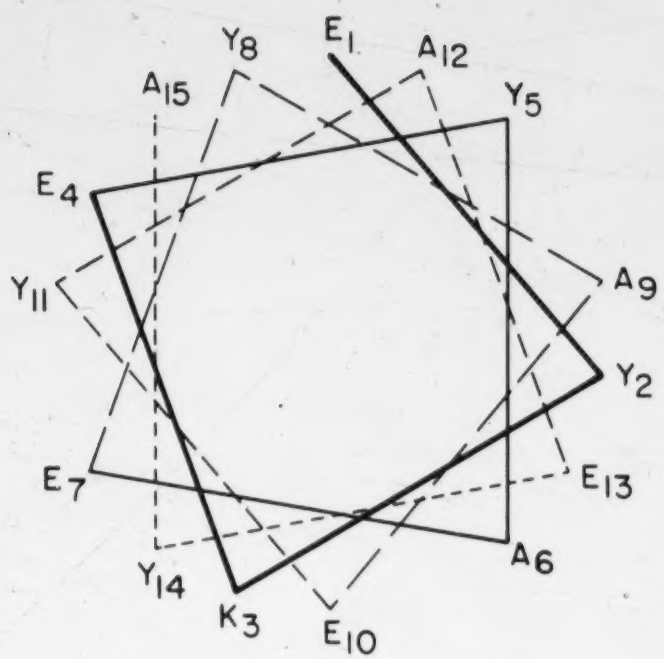

Figure 3.5. Two-dimensional projection showing the first 15 amino acids of poly 18 modelled in an α-helical conformation. The molecule is viewed from the N terminal, looking down the axis of the helix: amino acid residues are numbered according to their order in the molecule and should be visualized as forming a spiral entering the page. Each apex gives the approximate orientation of the amino acid side chain at that position.

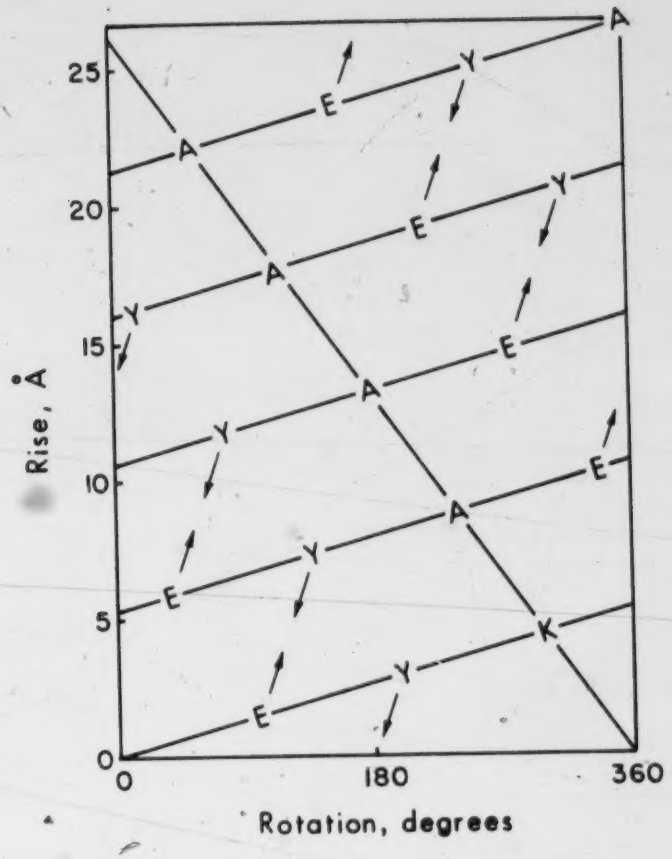

Figure 3.6. Two dimensional projection of poly 18 modelled in an α-helical conformation. The α-helix is visualized as a cylinder which has been cut down one side, opened and laid flat. The side chains of the amino acid residues (identified by the single letter code as in Table 3.1) project out of the page. The diagonal extending from the top left hand corner to the bottom right hand corner represents the alanine ridge discussed in the text. Arrows show the interaction between Tyr and Glu side chains which are thought to draw these residues away from the alanines.

REFERENCES

1. **Unanue, E.R.** 1984. Antigen-presenting function of the macrophage. *Ann.Rev.Immunol. 2*:395-428.

2. **Grey, H.M., and R. Chesnut.** 1985. Antigen processing and presentation to T cells. *Immunol.Today 6*:101-106.

3. **Haskins, K., J. Kappler, and P. Marrack.** 1984. The major histocompatibility complex-restricted antigen receptor on T cells. *Ann.Rev.Immunol. 2*:51-66.

4. **Schwartz, R.H.** 1985. T lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. *Ann.Rev.Immunol. 3*:237-261.

5. **Babbitt, B.P., P.M. Allen, G. Matsueda, E. Haber, and E.R. Unanue.** 1985. Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature 317*:359-361.

6. **Buus, S., S. Colon, C. Smith, J.H. Freed, C. Miles, and H.M. Grey.** 1986. Interaction between a "processed" ovalbumin peptide and Ia molecules. *Proc.Natl.Acad.Sci.USA 83*:3968-3971.

7. **Heber-Katz, E., D. Hansburg, and R.H. Schwartz.** 1983. The Ia molecule of the antigen-presenting cell plays a critical role in immune response gene regulation of T cell activation. *J.Mol.Cell.Immunol. 1*:3-14.

8. **Ziegler, H.K., and E.R. Unanue.** 1982. Decrease in macrophage antigen catabolism caused by ammonia and chloroquine is associated with inhibition of antigen presentation to T cells. *Proc.Natl.Acad.Sci.USA 79*:175-178.

9. **Germain, R.N.** 1986. The ins and outs of antigen processing and presentation. *Nature 322*:687-689.

10. **Falo, L.D., K. Sullivan, B. Benacerraf, M.F. Mescher, and K.L. Rock.** 1985. Analysis of antigen presentation by metabolically inactive accessory cells and their isolated membranes. *Proc.Natl.Acad.Sci.USA 82*:6647-6651.

11. **Chesnut, R.W., S.M. Colon, and H.M. Grey.** 1982. Antigen presentation by normal B cells, B cell tumors, and macrophages: functional and biochemical comparison. *J.Immunol. 128*:1764-1768.

12. **Thomas, D.W., J.L. Schauster, M.D. Hoffman, and G.D. Wilner.** 1985. Nonrandom catabolism of proteins in the

formation of antigenic peptide fragments. *J.Immunol. 135*:1259-1263.

13. **Thomas, D.W., M.J. Solvay, D.N. Irani, and R. Nairn.** 1985. Importance of the COOH terminal of angiotensin in antigenicity and in the formation of an antigen-containing complex with cellular membrane structures. *J.Immunol. 135*:4086-4089.

14. **Shimonkevitz, R., J. Kappler, P. Marrack, and H. Grey.** 1983. Antigen recognition by H-2-restricted T cells. *J.Exp.Med. 158*:303-316.

15. **Allen, P.M., and E.R. Unanue.** 1984. Differential requirements for antigen processing by macrophages for lysozyme-specific T hybridomas. *J.Immunol. 132*:1077-1079.

16. **Streicher, H.I., I.J. Berkower, M. Busch, F.R.N. Gurd, and J.A. Berzofsky.** 1984. Antigen conformation determines processing requirements for T cell activation. *Proc.Natl.Acad.Sci.USA 81*:6831-6835.

17. **Watts, T.M., A.A. Brian, J.W. Kappler, P. Marrack, and H.M. McConnell.** 1984. Antigen presentation by supported planar membranes containing affinity-purified I-A^d. *Proc.Natl.Acad.Sci.USA 81*:7564-7568.

18. **Buus, S., and O. Werdelin.** 1986. Oligopeptide antigens of the angiotensin lineage compete for presentation by paraformaldehyde-treated accessory cells to T cells. *J.Immunol. 136*:459-465.

19. **Kovac, Z., and R.H. Schwartz.** 1985. The molecular basis of the requirement for antigen processing of pigeon cytochrome c prior to T cell activation. *J.Immunol. 134*:3233-3240.

20. **Lee, K.C., M. Wong, and D. Spitzer.** 1982. Chloroquine as a probe for antigen processing by accessory cells. *Transplantation 34*:150-153.

21. **Fotedar, A., M. Boyer, W. Smart, J. Widtman, E. Fraga, and B. Singh.** 1985. Fine specificity of antigen recognition by T cell hybridoma clones specific for poly-18: a synthetic polypeptide antigen of defined sequence and conformation. *J.Immunol. 135*:3028-3033.

22. **Barton, M., B. Singh, and E. Fraga.** 1977. Synthetic polypeptide antigens of defined geometry. *J.Am.Chem.Soc. 99*:8491-8498.

23. **Singh, B., E. Fraga, and M. Barton.** 1978. Characterization and genetic control of the immune response

to synthetic polypeptide antigens of defined geometry. *J.Immunol. 121*:784-789.

24. **Singh, B., K.-C. Lee, E. Fraga, A. Wilkinson, M. Wong, and M. Barton.** 1980. Minimum peptide sequences necessary for priming and triggering of humoral and cell mediated immune responses in mice: use of synthetic peptide antigens of defined structure. *J.Immunol. 124*:1336-1343.

25. **Glimcher, L.G., T. Hamano, R. Asofsky, E. Heber-Katz, S. Hedrick, R.H. Schwartz, and W.E. Paul.** 1982. I region-restricted antigen presentation by B cell-B lymphoma hybridomas. *Nature 298*:283-284.

26. **Morinaga, T., A. Fotedar, B. Singh, T.G. Wegmann, and T. Tamaoki.** 1985. Isolation of cDNA clones encoding a T-cell receptor beta chain from a beef insulin specific hybridoma. *Proc.Natl.Acad.Sci.USA 82*:8163-8167.

27. **Smart, W., A. Fotedar, M. Boyer, E. Fraga, J. Lauzon, and B. Singh.** 1986. Analysis of the molecular determinant recognized by a beef insulin specific T cell hybridoma. In *The immunology of Diabetes mellitus*. M.A. Jaworski, G.D. Molnar, R.V. Rajotte and B. Singh, eds. Excerpta Medica, Elsevier Science Publishers, Amsterdam, P.249.

28. **Heber-Katz, E., R.H. Schwartz, L.A. Matis, C. Hannum, T. Fairwell, E. Appella, and D. Hansburg.** 1982. The contribution of antigen presenting cell major histocompatibility complex gene products to the specificity of antigen induced T cell activation. *J.Exp.Med. 155*:1086-1099.

29. **Watson, J.** 1979. Continuous proliferation of murine antigen-specific helper T lymphocytes in culture. *J.Exp.Med. 150*:1510-1519.

30. **Hedrick, S.M., L.A. Matis, T.T. Hecht, L.E. Samelson, D.L. Longo, E. Heber-Katz, and R.H. Schwartz.** 1982. The fine specificity of antigen and Ia determinant recognition by T cell hybridoma clones specific for pigeon cytochrome c. *Cell 30*:141-152.

31. **Pincus, M.R., F. Gerewitz, R.H. Schwartz, and H.A. Scheraga.** 1983. Correlation between the conformation of cytochrome c peptides and their stimulatory activity in a T-lymphocyte proliferation assay. *Proc.Natl.Acad.Sci.USA 80*:3297-3300.

32. **DeLisi, C., and J.A. Berzofsky.** 1985. T-cell antigenic sites tend to be amphipathic structures. *Proc.Natl.Acad.Sci.USA 82*:7048-7052.

33. **Berkower, I., G.K. Buckenmeyer, and J.A. Berzofsky.** 1986. Molecular mapping of a histocompatibility-restricted immunodominant T cell epitope with synthetic and natural peptides: implications for T cell antigenic structure. *J.Immunol. 136*:2498-2503.

34. **Chothia, C., M. Levitt, and D. Richardson.** 1977. Structure of proteins: packing of alpha-helices and pleated sheets. *Proc.Natl.Acad.Sci.USA 74*:4130-4134.

35. **Chothia, C., M. Levitt, and D. Richardson.** 1981. Helix to helix packing in proteins. *J.Mol.Biol. 145*:215-250.

36. **Ramachandran, J., A. Berger, and E. Katchalski.** 1971. Synthesis and physiochemical properties in aqueous solution of the sequential polypeptide poly(Tyr-Ala-Glu). *Biopolymers 10*:1829-1851.

CHAPTER 4

Identification of critical residues in peptide antigens recognized by T cell hybridomas A.1.1 and B.1.1

T lymphocytes bearing CD4 recognize antigen in the context of Class II Major Histocompatibility Complex encoded proteins (Ia), present on the surface of antigen presenting cells [1]. There is functional evidence that antigen and Ia must interact to induce T cell stimulation [1], as well as direct evidence that Ia binds antigen [2,3]. These findings strongly support a model of T cell activation requiring the formation of a ternary complex between the T cell receptor, antigen and Ia [4]. The demonstration that antigens compete with each other for binding to soluble Ia [5,6] and for activation of T cells in functional assays [7] supports the idea that Class II proteins have a single binding site for antigen.

Peptide antigens recognized by T cells vary in length from 7 to 15 amino acids [8]. Among the residues which make up the antigen, some are found to play a critical role while others seem to be dispensable or replaceable by unrelated amino acids. Critical residues of antigen have been identified in a number of systems [9-13] and have been assigned T cell interaction or Ia interaction roles

[4,12,14,15]. In addition, evidence that residues of antigen interact with each other in a manner critical for T cell recognition has been obtained recently in the antigen EYK(EYA)$_4$, thereby suggesting a third role for critical residues (Chap. 6). The requirement for a great variety of T cell antigens to bind to a limited number of Ia molecules puts some constraints on the diversity of sequences which can interact with the Class II molecule and, as a result, many peptide antigens appear to share a common motif [6,15-17].

We reported earlier on the fine specificity of poly 18 reactive I-A^d-restricted T cell hybridomas generated in this laboratory [18]. Poly 18, i.e. poly EYK(EYA)$_5$, is a synthetic polypeptide antigen of defined sequence and alpha-helical conformation [19-21], for which Balb/c (H-2^d) mice are responders and C57Bl/10 (H-2^b) and C3H (H-2^k) are non-responders. A panel of synthetic peptides were used for these specificity studies that revealed two groups of hybridomas, exemplified by clones A.1.1 and B.1.1, which are further examined in this report.

We identified the critical residues of the 15 amino acid peptide EYK(EYA)$_4$ which stimulates A.1.1 and B.1.1, and of (EYA)$_5$ which activates B.1.1, using related peptide analogs with single amino acid substitutions. In addition, activation studies of A.1.1 and B.1.1 using substituted peptides presented by normal and glutaraldehyde-fixed APC showed no requirement for antigen processing in the response to the peptides tested. Further investigations to determine the role

of the critical residues, using competition experiments, are reported in chapter 5.

MATERIALS AND METHODS

Animals. BALB/cCr mice were bred at the Ellerslie Animal Farm of the University of Alberta, or were purchased from The Jackson Laboratory, Bar Harbor, ME.

Antigens. The synthetic strategy for poly-18 and the various related peptides was essentially as described previously [18,19]. In brief, peptides were prepared by the Merrifield solid phase peptide synthesis technique [22]. The synthesis was performed on a Beckman 990C Peptide Synthesizer. All amino acids were protected at the α-amino position with the BOC group and the following side chain blocking groups were used: Glu(O-benzyl), Tyr(2-bromobenzoxycarbonyl), Lys(2-chlorobenzyloxycarbonyl), Lys(2,4-dinitrophenyl) and Arg(tosyl). Dicyclohexylcarbodiimide was used as the activating agent and double couplings of 2 h each were performed at each step with 2.5 equivalents of BOC amino acids. The BOC groups were removed at each cycle of the synthesis by treatment with 25% trifluoroacetic acid (TFA) in methylene chloride (v/v) for 20 minutes and neutralization was carried out by treatment with 5% diisopropylethylamine in methylene chloride (v/v).

The cleavage of the peptide from the resin support and removal of the blocking groups was carried out in hydrofluoric acid (HF) at 0°C for 2 h with 10% anisol and 0.1% ethane dithiol as scavengers. After removal of the HF

under vacuum, the resin was washed with ether and the peptide extracted with TFA. The TFA was evaporated to a small volume and the peptide was precipitated with ether. After precipitation the peptides were dissolved in water and lyophylized. The crude material was purified by HPLC. Samples were dissolved in water at pH 7.2 (10% NH_4OH) and applied to a C-18 reversed phase preparative SynChropak RP-P column. Peptides were eluted with a linear gradient from water to acetonitrile (1.37% acetonitrile per minute). Fractions were pooled and lyophilized. Amino acid analysis was performed on the purified peptides by using a Beckman System 6300 amino acid analyser. Samples were hydrolysed in 6N HCl containing 0.1% phenol, in sealed evacuated tubes for 20 h at 110°C. All peptides gave acceptable ratios of the various amino acids, For functional assays, peptides were dissolved in saline by adjusting pH to 7.2 with 0.1N NaOH, were sterilized by filtration through a 0.22 μm filter, and were stored at -20°C.

T cell hybridomas. Poly 18 reactive, I-A^d restricted T cell hybridomas were generated in this laboratory and have been previously described [18].

Antigen presenting cells. For antigen presentation, cells from the I-$A^{d/k}$/I-$E^{d/k}$ bearing B cell hybridoma TA3 [23], were added at 1-2.5 x 10^4 cells per well. For glutaraldehyde fixation, cells were washed twice with Hepes buffered balanced salt solution (HBBSS) and 5 x 10^6 cells/ml in HBBSS were treated with a final concentration of 0.05% of

glutaraldehyde (Fisher Scientific), as described [24]. After half a minute at room temperature, fixation was stopped by addition of an equal volume of 0.2 M lysine (Sigma) in HBBSS. Cells were washed twice in HBBSS and stored at 4°C for one week before use. Efficacy of the glutaraldehyde treatment was determined by [^{3}H]-thymidine uptake by the fixed cells in a 24 h incubation at 37°C. Uptake by fixed cells was less than 1% of normal cell uptake.

IL 2 assay. T hybridoma cells (5 x 10^4) were co-cultured with 1-2.5 x 10^4 normal or 5 x 10^4 glutaraldehyde-fixed TA3 cells, in the presence or absence of antigen in 250 µL of culture medium (RPMI 1640 containing 10 mM HEPES, 2 mM glutamine, 5 x 10^{-5} M 2-mercaptoethanol, 10% heat inactivated fetal calf serum, and 1 U/mL penicillin-streptomycin (Gibco). After 24 to 42 hours, supernatants were collected and were assayed for IL-2 content in a secondary culture by using the IL-2 dependent clone CTL-L (kindly provided by Dr. T. Delovitch, University of Toronto, Canada). Cells (10^4) were cultured for 40 h with 12.5% primary supernatant, and the degree of stimulation was measured by the incorporation of [^{3}H]-thymidine (40 kBq/well). Amount of primary supernatant in culture was determined to stimulate in the linear portion of the CTL-L dose response curve.

RESULTS

Antigen fine specificity of clone A.1.1. We previously reported that recognition of poly 18 [poly EYK(EYA)$_5$] by clone A.1.1 was aimed at the lysine containing segment of the polypeptide [18]. As Table 4.1 shows, this clone responded to EYK(EYA)$_4$ but not to (EYA)$_5$. These results identified Lys at position 3 of the 15 amino acid peptide EYK(EYA)$_4$ as a critical residue for the stimulation of A.1.1. (For convenience, amino acids were numbered 1-15 from N-terminus to C-terminus.). Our previous analysis also showed that Ala 9 was critical [18] and this was confirmed by the lack of response to EYKEYAEYLEYAEYA, in which Ala 9 is substituted by leucine (Table 4.2). In contrast, Ala 15 was not found to be critical.

In order to identify other functionally important residues in the antigen and evaluate their role, substituted peptides were synthesized and tested for their ability to stimulate T hybrid A.1.1 to release IL-2.

Tyr 8, Glu 10 and Tyr 11 are critical residues of EYK(EYA)$_4$ for the activation of A.1.1. The role of glutamic acid and tyrosine residues in EYK(EYA)$_4$ was addressed by substituting each of these residues with alanine. As shown in Table 4.1, substituting positions 4, 5 and 7 had some effect on the potency of the resulting peptides, but suggested that these

residues were not critical for the stimulation of A.1.1. In fact, dose response curves showed that peptides with substitutions at positions 4 and 7, EYKAYA$(EYA)_3$ and EYREYAAYA$(EYA)_2$, were more potent than the parent antigen EYK$(EYA)_4$ (Figure 4.1). Although Glu 4 and Tyr 5 could be substituted individually without destroying the response, replacing both residues with alanine resulted in complete loss of antigenicity (Table 4.3). Substitutions at positions 1 and 2, and at positions 13 and 14 also showed some effect on potency, but did not indicate a critical role for these residues. In contrast, substitutions at Tyr 8, Glu 10 and Tyr 11 abolished the response of A.1.1. Multiple substitutions involving these 3 residues also gave rise to non-stimulatory peptides (Table 4.3). We conclude from these results that Tyr 8, Glu 10 and Tyr 11 are critical residues for the activation of clone A.1.1.

Position 12 is critical for A.1.1 activation. To assess the role of alanine residues, these were substituted with various amino acids and resulting peptides were tested for their ability to stimulate clone A.1.1. Lysine was one of the substituting amino acids chosen because of its importance in the immunogen poly 18. When Ala 6, Ala 12 and Ala 15 in EYK$(EYA)_4$ were substituted by Lys, a minimum decrease in potency was observed with the substitution at position 15. A substantial decrease occurred with the substitution at position 6 (Table 4.2). However, the substitution at

position 12 abolished the response of clone A.1.1 (Table 4.2), suggesting a critical role for this position. We conclude from these and the above results that Lys 3, Tyr 8, Ala 9, Glu 10, Tyr 11 and Ala 12 are critical residues for the activation of clone A.1.1.

Antigen processing of peptides is not required for A.1.1 response. Processing of antigen by the antigen-presenting cell has been shown to influence the response of T cells [25,26]. To assess the effect of processing in our stimulation assays, antigen-presenting cells were fixed with glutaraldehyde before culture with antigen. Figure 4.1B and Tables 4.1 and 4.2 show that peptides behaved in a similar fashion when presented by normal or glutaraldehyde-treated APC. This confirmed that results obtained with substituted peptides were not due to processing events indirectly altering the responses but to the direct effect of the amino acid substitutions on recognition.

Response of clone B.1.1 to poly 18-related peptides. The response of clone B.1.1 to poly 18 is preferentially directed to a 15 amino acid determinant [18] composed of the sequences $(EYA)_5$ or $EYK(EYA)_4$ (Fig. 4.2). However, this hybridoma also responded to $(EYA)_4EYK$. These findings suggested that amino acids at positions 3 and 15 were not critical for antigen recognition, although they affected the magnitude of the stimulation. A very low response could also be obtained to

$A(EYA)_4$ [27], (Chap. 3) suggesting that residues 1 and 2 were not essential for recognition but played a role in potency of the antigen.

Glu 7, Tyr 8 and Tyr 11 are critical residues of $EYK(EYA)_4$ for activation of B.1.1. Applying the strategy used with clone A.1.1 to identify functionally important residues in the antigen $EYK(EYA)_4$, substituted peptides were tested for their ability to stimulate clone B.1.1 to release IL-2. As shown in Table 4.1, substituting position 4 with alanine had little effect on the ability of the peptide to stimulate clone B.1.1, suggesting that Glu 4 is not a critical residue in $EYK(EYA)_4$. Substitutions of Tyr 5 and Glu 10 resulted in a large decrease in potency but did not indicate a critical role for these residues. In contrast, substitutions at Glu 7, Tyr 8 and Tyr 11 abolished the response of clone B.1.1. Substitutions at positions 13 and 14 did not reveal a critical role for these positions as the peptide $(EYA)_4AAK$ could elicit a response from B.1.1 (Table 4.3). We conclude from these results that Glu 7, Tyr 8 and Tyr 11 are critical residues of $EYK(EYA)_4$ for the activation of clone B.1.1.

Glutamic acid and tyrosine residues were also substituted in the antigenic peptide $(EYA)_5$, yielding a similar pattern of reactivity to the modified peptides, except in the case of Glu 7. Peptides with Glu 4 and Tyr 5 substituted individually retained activity but lost it when both residues were replaced by Ala (Table 4.3). Residues Tyr

8 and Tyr 11 were found to be critical in this antigen as in EYK(EYA)$_4$, and multiple substitutions involving these positions also gave non-stimulatory peptides (Table 4.3).

Positions 6, 9 and 12 are critical for B.1.1 activation. The role of alanine residues was also assessed using substituted peptides. When the alanines in (EYA)$_5$ were substituted by Lys, a minimum decrease in potency was observed with the substitution at position 3 and an approximate tenfold decrease occurred with the substitution at position 15 (Figure 4.2). However, substitutions at positions 6, 9 and 12 abolished the response of clone B.1.1 (Table 4.2), suggesting a critical role for these positions. This was confirmed by substitutions in peptide EYK(EYA)$_4$ as EYKEYK(EYA)$_3$ and EYK(EYA)$_2$EYKEYA both failed to stimulate B.1.1.

The importance of Ala 9 was further illustrated by the following observations. Double substitutions with lysine at positions 3 and 15, generating EYK(EYA)$_3$EYK, had a profound effect on the response at an antigen dose of 40 μM. This effect could be partially overcome by increasing the antigen dose (Figure 4.2) or by derivatizing one or the other lysine residue with DNP (Table 4.2), suggesting that the two positive charges introduced by the lysine residues were responsible for the effect of this peptide on the response of clone B.1.1. Surprisingly, this negative influence of Lys 3 and Lys 15 could be dramatically reversed by substituting Ala

9 by leucine to obtain EYKEYAEYLEYAEYK (Table 4.2 and Figure 4.2). Glu and Arg substitutions at this position did not rescue the response. This result emphasizes that position 9 plays a crucial role in the stimulation of B.1.1. We conclude from these results that Ala 6, Ala 9 and Ala 12 are critical residues for B.1.1.

Antigen processing of peptides is not required for the response of B.1.1. To determine if antigen processing was necessary for the stimulation of B.1.1, peptides were presented by glutaraldehyde-fixed antigen presenting cells. Figure 4.2B and Table 4.1 show that most peptides behaved in a similar fashion when presented by normal or glutaraldehyde treated APC, suggesting that the effects of amino acid substitutions on B.1.1 responses were not due to processing. However, we made two unusual findings. The peptide where Tyr 5 was replaced by Ala showed very low stimulatory activity when normal APC were used (Table 4.2). In contrast, a moderate response could be obtained with this peptide when glutaraldehyde-fixed APC were used. Likewise, substitution of Glu 10 reduced responsiveness considerably, again more so with normal than with fixed APC.

DISCUSSION

In this report, we analyzed the response of T cell hybridomas A.1.1 and B.1.1 to substituted peptides related to the immunogenic peptide EYK(EYA)$_4$, which stimulates both clones, and to peptide (EYA)$_5$, which activates B.1.1. We identified 6 critical residues for A.1.1: Lys 3, Tyr 8, Ala 9, Glu 10, Tyr 11, Ala 12; and 6 critical residues for B.1.1: Ala 6, Glu 7, Tyr 8, Ala 9, Tyr 11 and Ala 12 (Figure 4.3). Using glutaraldehyde-fixed antigen presenting cells, we showed that A.1.1 and B.1.1 do not require processed antigen for activation. However, two peptides were presented more efficiently by fixed cells than by normal cells.

The critical residues of EYK(EYA)$_4$ for both hybridomas are clustered in the middle of the antigen, between residues 6 and 12. Only Lys 3, which is critical for A.1.1 stimulation, lies outside this region, in the N-terminal third of the antigen. This clustering of critical residues is also observed in several other antigens such as cytochrome c [22,28], hen egg lysozyme [14], and ovalbumin [15]. This may reflect a general property of T cell antigens dictated by the requirement to interact both with the T cell receptor and Ia.

Four residues of EYK(EYA)$_4$ are critical for both A.1.1 and B.1.1: Tyr 8, Ala 9, Tyr 11 and Ala 12. It has been suggested that MHC-binding peptides which stimulate T cells share a common structural motif [16,17], consisting of two or

three hydrophobic residues flanked by charged or polar amino acids. Tyr 8 and Ala 9 are good candidates to form the hydrophobic region of this motif, and are flanked by charged residues, Glu 7 and Glu 10, in good agreement with the motif proposed by Rothbard and Taylor for this antigen [16].

Most T cells respond to a processed form of the native immunogen which elicits them [25,29]. We have shown [27], (Chap. 3), that poly 18 as well as minimum peptides derived from it are presented to A.1.1 and B.1.1 without requiring processing. Likewise, the substituted peptides that we generated to identify critical residues in the antigen were presented without processing. However, two amino acid substitutions produced a peculiar effect. The replacement of Tyr 5 and Glu 10 by alanine rendered the substituted peptides less stimulatory on normal APC; but with B.1.1, these regained potency when presented by glutaraldehyde-fixed cells. A possible explanation for this finding is that these substitutions introduce a processing site in the antigen, making it vulnerable to modification by the APC. However, this modification would have to affect the antigen in a way that can be recognized by B.1.1 but not by A.1.1. A more likely explanation is that the glutaraldehyde-modified Ia displays the substituted peptides in a way that selectively enhances the efficiency of interaction with B.1.1 but not with A.1.1. This would be in keeping with our earlier findings [27], (Chap. 3), that B.1.1 is more sensitive than

$A_\alpha 1.1$ to the effect of glutaraldehyde on the presenting ability of APC.

Another intriguing set of observations is the number and kinds of substitutions which can be introduced in the antigens and be tolerated in T cell recognition. For example, B.1.1 responds well to $(EYA)_5$ and $EYK(EYA)_4$, poorly to $EYK(EYA)_3EYK$ and very well to EYKEYAEYLEYAEYK. Peptides $(EYA)_5$ and EYKEYAEYLEYAEYK, which are both stimulatory, have 3 amino acid differences between them, two of which are non-conservative. On the other hand, $EYK(EYA)_3EYK$ and EYKEYAEYLEYAEYK only have one amino acid difference and it is conservative (Ala to Leu); nevertheless, they display widely divergent potencies. It seems that Leu 9 compensates for the negative effect of Lys 15 on the response. Conversely, Lys 15 is important in the recognition of EYKEYAEYLEYAEYK since its absence in EYKEYAEYLEYAEYA results in a drastic potency drop. Therefore, there appear to be functional interactions between amino acids within antigens. Similar observations, discussed in Chapter 6, have been made for A.1.1. These results show the flexibility of amino acid composition permitted in a T cell antigen. Rothbard et al. have introduced six substitutions in two 13 amino acid antigens without destroying their ability to stimulate the T cell clones to which they gave rise [12]. Guillet et al. have shown that a hybridoma specific for a 13 residue bacteriophage lambda cI peptide could respond to a substituted ovalbumin peptide with 9 amino acid differences [7]. This degeneracy of recognition

can be partly accounted for by the low specificity of the antigen-Ia interaction. Amino acid substitutions in the agretope are more readily tolerated due to the broad specificity of the Class II molecule. However, a corollary of this is that the effect of a substitution in the agretope of an antigen should be felt by all hybridomas which respond to this antigen. We observe that some substitutions are permitted by clone A.1.1 but not by clone B.1.1 and vice-versa. Based on the above, these substitutions must be affecting the epitopes which suggests that the degeneracy of recognition is also at the level of the TCR-antigen interaction.

It would appear as if T cell recognition of antigen were not directed at specific amino acids but at a combination of residues which together form a structure with the appropriate features to bind Ia and the T cell receptor. It may be that Class II restricted T cells are mainly concerned with recognizing Ia and that the role of antigen is to increase the affinity of that interaction enough to trigger the T cell. This increase may be small and may be provided by a diversity of amino acid sequences.

Some of the substituted peptides were found to be more potent than the parent peptide EYK$(EYA)_4$. These were EYKAYA$(EYA)_3$ and especially, EYKEYAAYA$(EYA)_2$, for hybridoma A.1.1, and EYKEYAEYLEYAEYK for B.1.1. Such heteroclytic responses have also been reported for substituted peptides of cytochrome c [9,22]. In Chapter 5, we further analyse the

response of A.1.1 to EYKEYAAYA$(EYA)_2$ and propose a role for the amino acid which has been substituted.

TABLE 4.1

Response of clones A.1.1 and B.1.1 to EYK(EYA)$_4$ and (EYA)$_5$, and to peptides with tyrosine and glutamate residues substituted by alanine

Antigens	IL-2 Response CPM (x 10^{-3})			
	A.1.1		B.1.1	
	Normal TA3	G.A. TA3	Normal TA3	G.A. TA3
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15				
None	0.3	4	1	1
E Y K E Y A E Y A E Y A E Y A	80	31	412	360
A	68	14	441	200
A	42	22	2.5	20
A	71	56	0.2	0.5
A	0.5	5	0.2	0.5
A	0.3	2	4	55
A	0.5	2	0.1	0.6
A	0.3	3	459	320
A A	0.4	2	223	263
A A	44	27	20	24
A A	0.7	2	18	52
A A	0.3	4	0.4	0.5
A A	0.6	3	21	43
A A	0.4	2	0.5	0.7
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15				

Cells were cultured in the presence of normal or glutaraldehyde-fixed TA3 as APC and peptides at 100 μM. Supernatants from 24 h cultures were tested for their ability to stimulate [^{3}H]-thymidine uptake by CTLL. Results represent the mean of triplicate cultures. S.D. were less than 15% of the mean.

TABLE 4.2

Response of clones A.1.1 and B.1.1 to peptides with alanine residues substituted by various amino acids

Antigen	Il-2 response[a] CPM (x 10^{-3})			
	A.1.1		B.1.1	
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15	Normal TA3	G.A. TA3	Normal TA3	G.A. TA3
None	2	2	3	3
E Y A E Y A E Y A E Y A E Y A	4	4	134	61
___K_____	61	35	103	31
_____K_____	61	13	5	3
_____K_____	5	4	5	4
_____K_____	4	5	4	3
___K___K_____	14	7	5	3
___K_____K_____	4	2	5	2
___K_____K	66	22	5	3
___K_____K	2	ND	99	ND
^(DNP)				
___K_____K(DNP)	33	15	67	68
___K_____L_____K	6	6	78	91
___K_____E_____K	5	6	3	3
___K_____R_____K	4	5	5	3
___K_____L_____	2	2	20	11
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15				

a. T hybridoma cells were cultured in the presence of TA3 as APC and peptides at 80 μM. Supernatants from 24 h cultures were tested for their ability to stimulate [^{3}H]-thymidine incorporation by CTL-L. Results represent the mean of duplicate cultures. Standard deviations were less than 15% of the mean.

TABLE 4.3

Response of clones A.1.1 and B.1.1 to peptides with multiple substitutions

Antigens	IL-2 Respnse CPM (x 10^{-3})	
	A.1.1	B.1.1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15		
None	1.3	2.8
E Y K E Y A E Y A E Y A E Y A	41	45
A A K	27	ND
A A K	25	ND
A A K	ND	26
A A K	1.5	2.5
A A K	1	2.9
A A K	1.2	2.2
A A A K	1.1	2.6
A A K	1.3	2.8
A A K	1	2.7
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15		

T hybridoma cells were cultured in the presence of TA3 as APC and peptides at 100 μM. Supernatants from 24 h cultures were tested for their ability to stimulate [^{3}H]-thymidine incorporation by CTL-L. Results represent the mean of duplicate cultures. Standard deviations were less than 15% of the mean.

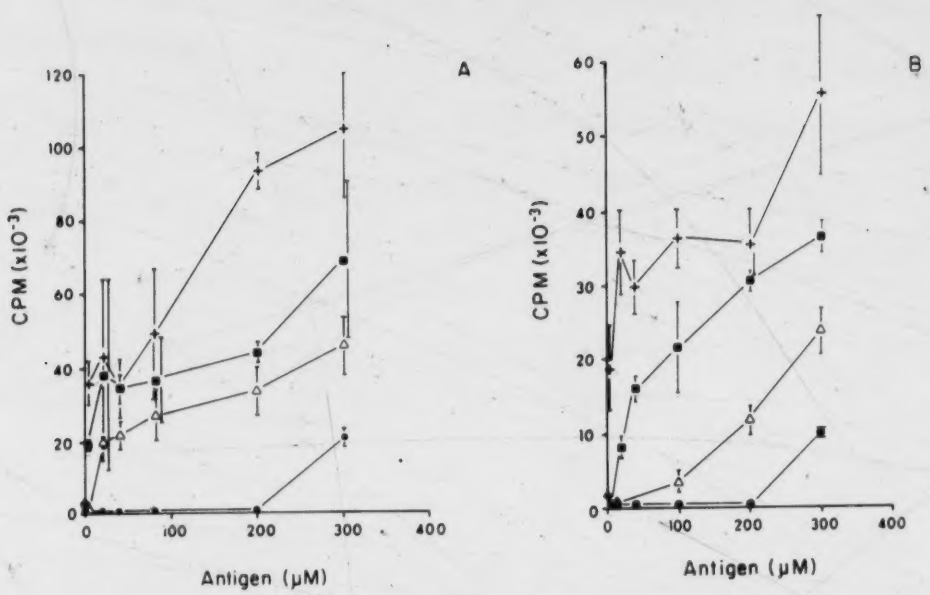

Figure 4.1. Dose response of T hybrid A.1.1 to peptide antigens presented by normal (panel A) and glutaraldehyde-fixed (panel B) TA3 cells. Each point represents the mean ± standard deviation of triplicate cultures. Antigens shown are: ◆: none, Δ: EYK(EYA)$_4$, ●:EYKEAA(EYA)$_3$, ■:EYKAYA(EYA)$_3$, +:EYKEYAAYA(EYA)$_2$.

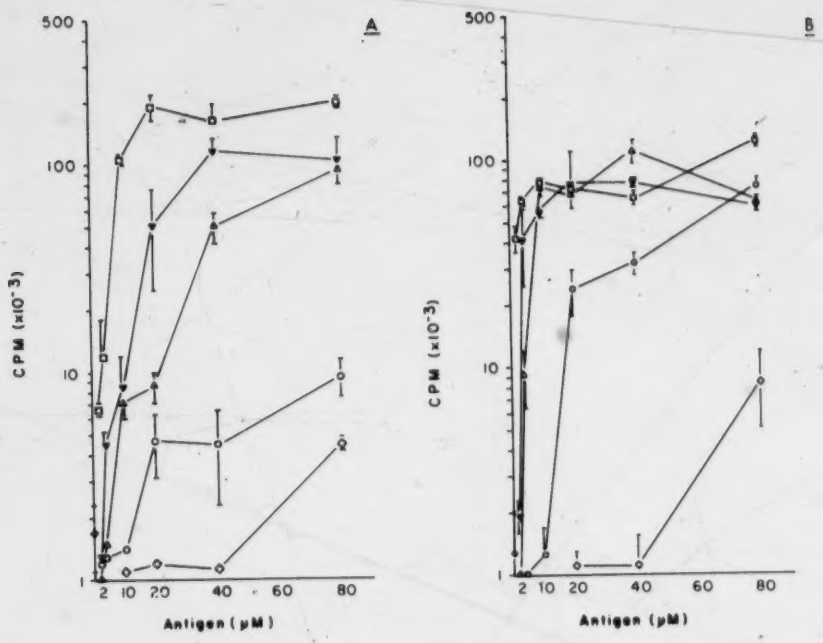

Figure 4.2. Dose response of T hybrid B.1.1 to peptide antigens presented by normal (panel A) and glutaraldehyde-fixed (panel B) TA3 cells. Each point represents the mean ± standard deviation of triplicate cultures. Antigens shown are: ◆: none, Δ:EYK(EYA)$_4$, ▼:(EYA)$_5$, □: EYKEYAEYLEYAEYK, ○:(EYA)$_4$EYK, ◇:EYK(EYA)$_3$EYK.

Figure 4.3. Critical amino acid residues of the antigenic peptide EYK(EYA)$_4$. Asterisks shown above and below the peptide sequence identify critical sites for the activation of clones A.1.1 and B.1.1, respectively.

REFERENCES

1. **Schwartz, R.H.** 1985. T lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. *Ann.Rev.Immunol. 3*:237-261.

2. **Babbitt, B.P., P.M. Allen, G. Matsueda, E. Haber, and E.R. Unanue.** 1985. Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature 317*:359-361.

3. **Buus, S., S. Colon, C. Smith, J.H. Freed, C. Miles, and H.M. Grey.** 1986. Interaction between a "processed" ovalbumin peptide and Ia molecules. *Proc.Natl.Acad.Sci.USA 83*:3968-3971.

4. **Heber-Katz, E., D. Hansburg, and R.H. Schwartz.** 1983. The Ia molecule of the antigen-presenting cell plays a critical role in immune response gene regulation of T cell activation. *J.Mol.Cell.Immunol. 1*:3-14.

5. **Buus, S., A. Sette, S.M. Colon, C. Miles, and H.M. Grey.** 1987. The relation between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunogenic peptides. *Science 235*:1353-1358.

6. **Guillet, J.-G., M.-Z. Lai, T.J. Briner, S. Buus, A. Sette, H.M. Grey, J.A. Smith, and M.L. Gefter.** 1987. Immunological self, nonself discrimination. *Science 235*:865-870.

7. **Guillet, J.-G., M.-Z. Lai, T.J. Briner, J.A. Smith, and M.L. Gefter.** 1986. Interaction of peptide antigens and class II major histocompatibility complex antigens. *Nature 324*:260-262.

8. **Livingstone, A.M., and C.G. Fathman.** 1987. The structure of T-cell epitopes. *Ann.Rev.Immunol. 5*:477-501.

9. **Solinger, A.M., M.E. Ultee, E. Margoliash, and R.H. Schwartz.** 1979. The T lymphocyte proliferative response to cytochrome c. I. Demonstration of a T cell heteroclitic proliferative response and identification of a single topographic antigenic determinant on pigeon cytochrome c whose immune recognition requires two complementing major histocompatibility complex linked response genes. *J.Exp.Med. 150*:830.

10. **Hansburg, D., C. Hannum, J.K. Inman, E. Appella, E. Margoliash, and R.H. Schwartz.** 1981. Parallel cross-reactive patterns of two sets of antigenically distinct

cytochrome c peptides: possible evidence for a presentational model of Ir gene function. *J.Immunol. 127*:1844.

11. **Watts, T.H., J. Gariepy, G. Schoolnik, and H.M. McConnell.** 1985. T cell activation by peptide antigen: Effect of peptide sequence and method of antigen presentation. *Proc.Natl.Acad.Sci.USA 82*:5480-5484.

12. **Rothbard, J.B., R.I. Lechler, K. Howland, V. Bal, D.D. Eckels, R. Sekaly, E.O. Long, W.R. Taylor, and J.R. Lamb.** 1988. Structural model of HLA-DR1 restricted T cell antigen recognition. *Cell 52*:515-523.

13. **Babbitt, B.P., G. Matsueda, E. Haber, E.R. Unanue, and P.M. Allen.** 1986. Antigenic competition at the level of peptide-Ia binding. *Proc.Natl.Acad.Sci.USA 83*:4509-4513.

14. **Allen, P.M., G.R. Matsueda, R.J. Evans, J.B.Jr. Dunbar, G.R. Marshall, and E.R. Unanue.** 1987. Identification of the T-cell and Ia contact residues of a T-cell antigenic epitope. *Nature 327*:713-715.

15. **Sette, A., S. Buus, S. Colon, J.A. Smith, C. Miles, and H.M. Grey.** 1987. Structural characteristics of an antigen required for its interaction with Ia and recognition by T cells. *Nature 328*:395-399.

16. **Rothbard, J.B., and W.R. Taylor.** 1988. A sequence pattern common to T cell epitopes. *EMBO J. 7*:93-100.

17. **Sette, A., S. Buus, S. Colon, C. Miles, and H.M. Grey.** 1988. I-Ad-binding peptides derived from unrelated protein antigens share a common structural motif. *J.Immunol. 141*:45-48.

18. **Fotedar, A., M. Boyer, W. Smart, J. Widtman, E. Fraga, and B. Singh.** 1985. Fine specificity of antigen recognition by T cell hybridoma clones specific for poly-18: a synthetic polypeptide antigen of defined sequence and conformation. *J.Immunol. 135*:3028-3033.

19. **Barton, M., B. Singh, and E. Fraga.** 1977. Synthetic polypeptide antigens of defined geometry. *J.Am.Chem.Soc. 99*:8491-8498.

20. **Singh, B., E. Fraga, and M. Barton.** 1978. Characterization and genetic control of the immune response to synthetic polypeptide antigens of defined geometry. *J.Immunol. 121*:784-789.

21. **Singh, B., K.-C. Lee, E. Fraga, A. Wilkinson, M. Wong, and M. Barton.** 1980. Minimum peptide sequences necessary for priming and triggering of humoral and cell

mediated immune responses in mice: use of synthetic peptide antigens of defined structure. *J.Immunol. 124*:1336-1343.

22. **Fox, B.S., C. Chen, E. Fraga, C.A. French, B. Singh, and R.H. Schwartz.** 1987. Functionally distinct agretopic and epitopic sites. Analysis of the dominant T cell determinant of moth and pigeon cytochromes c with the use of synthetic peptide antigens. *J.Immunol. 139*:1578-1588.

23. **Glimcher, L.G., T. Hamano, R. Asofsky, E. Heber-Katz, S. Hedrick, R.H. Schwartz, and W.E. Paul.** 1982. I region-restricted antigen presentation by B cell-B lymphoma hybridomas. *Nature 298*:283-284.

24. **Shimonkevitz, R., J. Kappler, P. Marrack, and H. Grey.** 1983. Antigen recognition by H-2-restricted T cells. *J.Exp.Med. 158*:303-316.

25. **Unanue, E.R.** 1984. Antigen-presenting function of the macrophage. *Ann.Rev.Immunol. 2*:395-428.

26. **Buus, S., and O. Werdelin.** 1986. A group-specific inhibitor of lysosomal cysteine proteinases selectively inhibits both proteolytic degradation and presentation of the antigen dinitrophenyl-poly-L-lysine by guinea pig accessory cells to T cells. *J.Immunol. 136*:452-458.

27. **Boyer, M., Z. Novak, A. Fotedar, and B. Singh.** 1988. Contribution of antigen processing to the recognition of a synthetic peptide antigen by specific T cell hybridomas. *J.Mol.Rec. 1*:99-106.

28. **Hansburg, D., T. Fairwell, R.H. Schwartz, and E. Appella.** 1983. The T lymphocyte response to cytochrome c. IV. Distinguishable sites on a peptide antigen which affect antigenic strength and memory. *J.Immunol. 131*:319.

29. **Grey, H.M., and R. Chesnut.** 1985. Antigen processing and presentation to T cells. *Immunol.Today 6*:101-106.

CHAPTER 5

Functional diversity of critical residues in peptide antigens recognized by T cell hybridomas A.1.1 and B.1.1

T lymphocytes bearing CD4 recognize antigen in the context of Class II Major Histocompatibility Complex encoded proteins (Ia), present on the surface of antigen presenting cells (reviewed in [1]). A large body of evidence indicates that this recognition event requires the formation of a ternary complex between the T cell receptor, antigen and Ia [2]. The demonstration that antigens compete with each other for binding to soluble Ia [3,4] and for activation of T cells in functional assays [5-7] supports the idea that Class II proteins have a single binding site for antigen.

Antigen competition studies have proven to be an attractive assay system because they offer the possibility to map which amino acid residues of antigen are T cell and Ia contact sites [8,9].

T cell recognition of peptides appears to require that the antigens present certain structural features. Several studies [1,8,10,11] support the hypothesis that T cells prefer antigens which can adopt secondary structure. Although α-helix has been proposed to be the preferred conformation, there is evidence for extended, β-sheet type structures as well [9].

In chapter 4, we identified the critical residues of EYK(EYA)$_4$ for the activation of T hybridomas A.1.1 and B.1.1 and the critical residues of (EYA)$_5$ for B.1.1. To accomplish this, single amino acid substitutions were introduced at every position of the antigens and the resulting peptides were tested for their ability to stimulate release of IL-2 from the hybridomas. In this chapter, we report on the use of substituted peptides with no stimulatory activity as competitive inhibitors of T hybrid activation. The purpose of these experiments was to gain information on the role of the critical residues which were identified. We hypothesized that substituted peptides which had the ability to compete would by necessity have an intact agretope, but would be lacking an epitope at the substituted residue. Conversely, those that did not compete would likely be missing an important Ia interaction site at the substituted residue. Therefore, these competition experiments would allow us to identify the T cell and Ia interaction sites on the antigen. We found that activation of the T hybrids could not be inhibited by peptides substituted at alanines or tyrosines or, in the case of A.1.1, at lysine. However, substitutions of glutamic acid residues generated good competitors suggesting that these residues were involved in the epitope. Peptides substituted at glutamic acid residues were also found to bind strongly to the APC, suggesting, in addition to their epitope role, an agretope role for these residues. Circular dichroism studies

showed in peptides derived from poly 18 an overall low propensity to adopt ordered secondary structure.

MATERIALS AND METHODS

Animals, antigens, T cell hybridomas, Antigen presenting cells, and *IL-2 assay* have been described in chapter 4.

Inhibition assay. A.1.1 or B.1.1 were incubated for 24 h with TA3 cells, the stimulating antigen EYK(EYA)$_4$ at 40 μM and competing peptides at the indicated doses. In some experiments with B.1.1, the stimulating antigen was (EYA)$_5$ at 10 μM. Supernatants were tested for the presence of IL-2 as described [12]. Percent inhibition was calculated as $\frac{\text{CPM control - CPM with competitor}}{\text{CPM control}} \times 100\%$. CPM control were those obtained with the stimulating antigens in the absence of competing peptide (mean of triplicate cultures). CPM with inhibitor were the mean of triplicate cultures determined to be significantly less than CPM control by paired t-test ($p<0.05$).

APC prepulsing with antigen. 5 x 10^5 TA3 cells were incubated in 200 μL of medium with antigen at various concentrations, for 2 h or 18 h. Cells were washed twice in RPMI and plated at 3-4 x 10^4 cells per microtiter well with 5 x 10^4 T hybridoma cells for 18 h at 37°C, in 200 μL of medium. Supernatants were tested for the presence of IL-2 as described [12].

Circular dichroism. The CD measurements were done on a Jasco J-500C spectropolarimeter with a DP-500N data processor at 25°C in a thermostatted cell holder. The samples were

scanned eight times using a .0103 cm cell from 260 to 180 nm. Peptides were analysed at a concentration of 0.5 mg/mL and at pH 7. The instrument was calibrated with d(+)10-camphor sulfonic acid at 290 nm and pantoyllactone at 219 nm. The ellipticity was calculated as follows:

$$[\theta] = \frac{\theta \times MRW}{10 \times c \times l}$$

where θ is the observed ellipticity in millidegrees, MRW is the mean residue weight, c is the concentration of sample in mg/mL, l is the pathlength of the cell in cm. Units of ellipticity are degree-cm^2/decimole. Estimates of amount of secondary structure (in percent) were obtained using the Provencher and Glöckner program of Contin 1 [13].

RESULTS

Competitive inhibition of antigenic peptides by non-stimulatory peptides. Poly 18-related peptides with single and multiple amino acid substitutions were synthesized and tested for their ability to stimulate T hybridomas A.1.1 and B.1.1 (chapter 4). Peptides which failed to stimulate were used in a functional competition assay to inhibit the activation of A.1.1 and B.1.1 by the antigenic peptides EYK(EYA)$_4$ and (EYA)$_5$. Experiments were first performed to establish that inhibition by negative peptides was competitive. As Figure 5.1 shows, non-stimulatory peptides EYKEYAEYAAYAEYA and EYAEYAAAAEYAEYK could prevent activation of A.1.1 and B.1.1, respectively, in a dose dependent manner. Their effect could be overcome by increasing the dose of stimulatory peptide, indicating that inhibition was indeed competitive and was not due to a non specific toxic effect of the high doses of peptide used.

A.1.1 response to EYK(EYA)$_4$ is not inhibited by peptides with Lys or Tyr substituted by Ala. Non stimulatory peptides differing from EYK(EYA)$_4$ at a single amino acid position were tested for their ability to competetively inhibit activation of A.1.1 by the antigen. As Figure 5.2 shows, peptides with substitutions at Lys 3, Tyr 8 and Tyr 11 were unable to compete with EYK(EYA)$_4$. Based on our working hypothesis,

these findings would indicate an Ia interaction role for these residues. In light of this, however, the inability of $(EYA)_5$ (Lys 3 substituted by Ala) to compete was unexpected since this peptide stimulates B.1.1 and therefore possesses an appropriate Ia interaction site.

A.1.1 response to $EYK(EYA)_4$ is inhibited by peptides with Glu substituted by Ala. In contrast to the above, we found that EYKEYAEYAAYAEYA, bearing an Ala at position 10, was inhibitory (Fig. 5.2). Since, to compete, this peptide would require an intact Ia interaction site, we surmised that the substitution was affecting the peptide's ability to be recognized by the T cell. Thus, this result suggested that the substituted amino acid Glu 10 was involved in T cell interaction.

Competition by peptides with multiple amino acid substitutions. Peptides with more than one amino acid substitution were also tested on A.1.1 (Figure 5.3). Among these, EYKEYAEYLEYAEYK, $(EYA)_2EYK(EYA)_2$, and $(EYA)_3EYKEYA$ had no inhibitory effect, suggesting that they lacked an Ia interaction site. In the case of the first two peptides, this would be at position 9 and, in the case of the third at position 12. $(EYA)_2EAA(EYA)_2$ and $(EYA)_3EAAEYA$ were also negative, and as such were consistent with their lysine containing counterparts $EYKEYAEAA(EYA)_2$ and $EYK(EYA)_2EAAEYA$. This finding further involved Tyr 8 and 11 in Ia interaction.

In contrast, $(EYA)_2AYA(EYA)_2$ and $(EYA)_3AYAEYA$ were found to compete out $EYK(EYA)_4$. From these results, it became apparent that competing peptides shared a common feature: all had a glutamic acid substituted by alanine. Indeed, this substitution was the only feature which distinguished the two competitive peptides $(EYA)_2AYA(EYA)_2$ and $(EYA)_3AYAEYA$, from the non-competitor $(EYA)_5$. This suggested that the substitutions at these positions might be influencing the competitive ability of the peptides and, consequently, be involved in Ia interaction. So it appeared as if Glu 10 in $EYK(EYA)_4$ was interacting both with the T cell receptor and with the class II molecule.

B.1.1 response to $EYK(EYA)_4$ and $(EYA)_5$ is not inhibited by peptides with substitutions at Tyr and Ala positions. Inhibition of B.1.1 stimulation was tested with $EYK(EYA)_4$ and $(EYA)_5$ as the stimulating antigens and negative peptides differing from the antigen by single and multiple substitutions. As Figures 5.4 and 5.5 show, the same peptides which failed to inhibit A.1.1 responses were found to have no effect on the stimulation of B.1.1 by $EYK(EYA)_4$ or $(EYA)_5$. These are peptides substituted at Tyr 8, Ala 9, Tyr 11 and Ala 12. In addition, $EYAEYK(EYA)_3$ did not compete, suggesting that Ala 6 was also involved in Ia interaction.

B.1.1 response to $EYK(EYA)_4$ and $(EYA)_5$ is inhibited by peptides with Glu substituted by Ala. The substituted peptide EYKEYAAYAEYAEYA was found to compete, which suggested that Glu 7 was involved in T cell interaction. However, consistent

with what was observed in A.1.1 inhibition experiments, the competing peptide had a glutamic acid replaced by an alanine, which pointed to an Ia interaction role as well for this position. As expected, this peptide also competed against $(EYA)_5$ for the activation of B.1.1 (Fig. 5.4). Among peptides with several substitutions, those able to compete were also found to have a glutamic acid replaced by alanine (Fig. 5.5). We conclude from these results that Glu 7 interacts with the T cell receptor and Ia.

EYKEYAAYA$(EYA)_2$ is an efficient APC binder. The observation that replacing glutamic acid residues at positions 7 and 10 by alanine correlated with good competition led us to assign an Ia interaction role to these residues. It followed from this that the substituted peptides should bind well to antigen presenting cells, a prediction which was testable using EYKEYAAYA$(EYA)_2$ because this peptide stimulates A.1.1 efficiently. Antigen presenting cells were prepulsed with EYK$(EYA)_4$ or EYKEYAAYA$(EYA)_2$ for 2 to 18 hours and washed. A.1.1 cells were added for an additional 18 h and their IL-2 production was measured. As Figure 5.6 shows, a short pulse at low antigen dose was sufficient to obtain maximum response from A.1.1 using EYKEYAAYA$(EYA)_2$, whereas a much higher dose was required to stimulate the T hybridoma with EYK$(EYA)_4$. These results indicate that EYKEYAAYA$(EYA)_2$ binds more efficiently to TA3 than EYK$(EYA)_4$ and this enhanced binding

can be traced to the alanine substitution at position 7, since this is the only difference between the peptides. Similar results were obtained using glutaraldehyde-fixed TA3 (data not shown), suggesting that processing of the antigen by the APC had no bearing on this effect.

Peptides of poly 18 have a low propensity to adopt secondary structure. Previous studies on poly 18 [14] and on the related polypeptide antigen poly EYA [15] have shown that these molecules adopt an α-helical conformation under physiological conditions. This suggested to us that the 15 amino acid peptides derived from these antigens might also have ordered secondary structure or that features of the substituted peptides such as the ability to competitively inhibit T hybridoma responses could be related to structure. We investigated this using CD spectroscopy. As shown in Figure 5.7, there is little evidence of ordered structure within the peptides in aqueous solution. In a solution containing 50% of the helix-inducing solvent trifluoroethanol, the spectra shift as a result of a slight increase of approximately 10% to 17% in α-helicity, as determined by the Provencher and Glockner method. However, overall the peptides show a low propensity to adopt secondary structure.

DISCUSSION

In this chapter, we investigated the role of the critical residues previously identified (chapter 4) in the antigens EYK(EYA)$_4$ which stimulates A.1.1 and B.1.1, and (EYA)$_5$ which can activate B.1.1. We used substituted peptides with no stimulatory activity to competetively inhibit activation of the T hybridomas by EYK(EYA)$_4$ and (EYA)$_5$. We found that negative peptides with substitutions of tyrosine and alanine were unable to compete with the stimulatory antigens. Thus peptides with substitutions at Tyr 8, Ala 9, Tyr 11 and Ala 12 did not compete for either clone A.1.1 or B.1.1, suggesting that these residues were important in Ia interaction. In addition, Lys 3 appeared to play a role in Ia interaction for A.1.1, whereas Ala 6 performed a similar function for B.1.1.

Substitutions of glutamic acid residues by alanine generated potent competitors. This suggested that Glu 7 and Glu 10 were involved in the epitopes recognized by B.1.1 and A.1.1, respectively. However, we also presented evidence that these residues played a role in Ia interaction. These findings are summarized in Figure 5.8.

We investigated the structural properties of a number of peptides using circular dichroism and found that these

molecules have a slight propensity to adopt ordered secondary structure in a non-aqueous environment.

This study was based on the hypothesis that the T cell receptor and Ia interaction sites of an antigenic peptide could be identified using non-stimulatory analogs of the antigen in a functional competition assay. We reasoned that analogs differing from the antigen by a single amino acid should competitively inhibit T hybrid stimulation if the amino acid substitution affected the T cell receptor interaction site. In contrast, a substitution affecting the Ia interaction site would generate a non-competing analog. This approach was used successfully to identify the T cell receptor and Ia interaction sites of hen egg lysozyme peptide HEL(52-61) [8]. In our system, four residues were found to interact with Ia in the stimulation of both T hybridomas A.1.1 and B.1.1. Only two residues appeared to interact with the T cell receptor, Glu 7 and Glu 10. The former was critical for activation of B.1.1 and the latter for A.1.1. However, both these residues also appeared to be involved in Ia interaction.

Similar observations have also been made with the ovalbumin fragment Ova 323-336, where three residues were found to have a dual role as both T cell receptor and Ia sites [9]. These findings suggest that T cell receptor and Class II interaction sites of antigen are not always independent [1]. An alternative explanation for the role of dual-acting residues is that they may have an indirect effect

on contact sites. Their main role may be to induce or stabilize a conformation which allows the T cell receptor and Ia interaction residues to be positioned appropriately. We will present evidence in chapter 6 that Lys 3 in the antigen $EYK(EYA)_4$ is critical for the activation of A.1.1 only when tyrosine is present at position 5. Substituting Tyr 5 by alanine removes the requirement for Lys 3 suggesting that the latter residue's role in the stimulation of A.1.1 is primarily to interact with Tyr 5. It is therefore possible that Glu 7 and Glu 10, which have all the appearances of contacting the T cell receptor and Ia, are actually acting indirectly. However, if this were the case, we would expect to find one or more other residues in the antigen which could interact with the T cell receptor. But there are no other candidate residues for this role, suggesting that Glu 7 and Glu 10 are likely involved in direct contact with the receptor.

In any case, this analysis shows the difficulties associated with attempts to assign unequivocal roles to specific amino acids of antigen. As we have discussed in chapter 4, it seems that there is considerable degeneracy in T cell recognition of antigen, not only in the case of poly 18 but also of other antigens [7,16]. The fact that a given T cell receptor can interact with a number of amino acid sequences suggests that the individual residues which make up the antigen are not as critical as the overall topography which they collectively generate, in association with the

Class II molecule. This may have important and useful implications for the generation of synthetic vaccines. It may be quite common that the best way to obtain effective immunization is not by administering the exact amino acid sequence as it is found in the native antigenic determinant but by using an engineered version which elicits the same clonotypes more efficiently. The ability to predict which substitutions to introduce in a peptide to increase its immunogenicity would be of great value. A starting point may be the sequence motif which has been proposed to form part of most T cell epitopes [4,17,18]. In the case of $EYK(EYA)_4$, the proposed motif comprises the 4 amino acid sequence EYAE [17]. Substituting the Glu at position 7 with Ala produces EYAA which now becomes, in the Rothbard model, a 5 amino acid motif: EYAAY. Thus the substitution creates a new motif with an increased hydrophobic region. In a sequence such as $EYK(EYA)_4$ with many charged residues, the enhanced hydrophobic stretch appears to provide a needed counterbalance for optimal interaction with Ia and the T cell receptor.

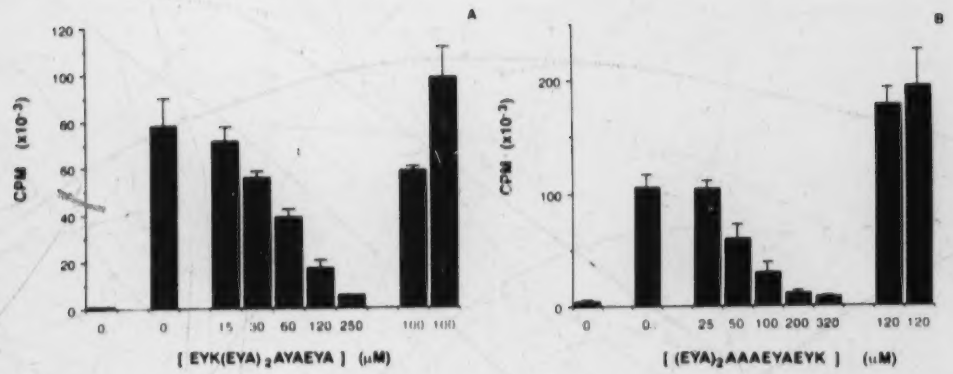

Figure 5.1. *(Panel A):* Competitive inhibition of A.1.1 response to EYK(EYA)$_4$ by substituted peptide EYKEYAEYAAYAEYA. The T hybrid was incubated for 24 h with TA3 cells, competing peptide EYKEYAEYAAYAEYA at the indicated doses and the antigen EYK(EYA)$_4$ at 40 μM except in column 1 where no antigen was used, and in columns 8 and 9 where 100 μM and 200 μM were used respectively. *(Panel B):* Competitive inhibition of B.1.1 response to (EYA)$_5$ by EYAEYAAAAEYAEYK. Culture conditions were as above, with the stimulating antigen dose at 10 μM except in columns 1, 8 and 9 (no antigen, 40 μM and 80 μM respectively). CPM represent the mean ± standard deviation of triplicate cultures.

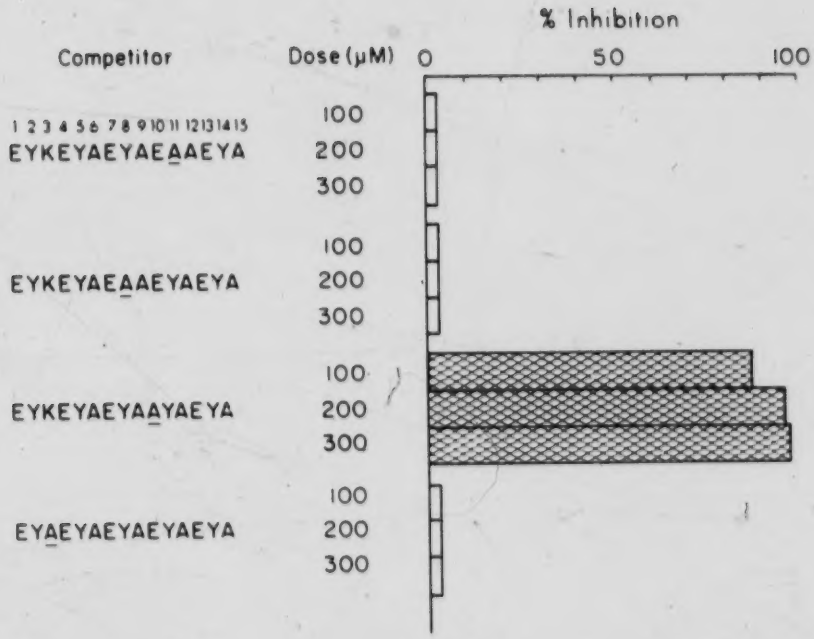

Figure 5.2. Competitive inhibition of A.1.1 response to EYK(EYA)$_4$ by peptides with single amino acid substitutions. T cell hybrids were incubated for 24 h with TA3 cells, stimulating antigen EYK(EYA)$_4$ at 40 μM and competing peptides at the indicated doses. Supernatants were tested for the presence of IL-2 and percent inhibition was calculated as described in Materials and Methods.

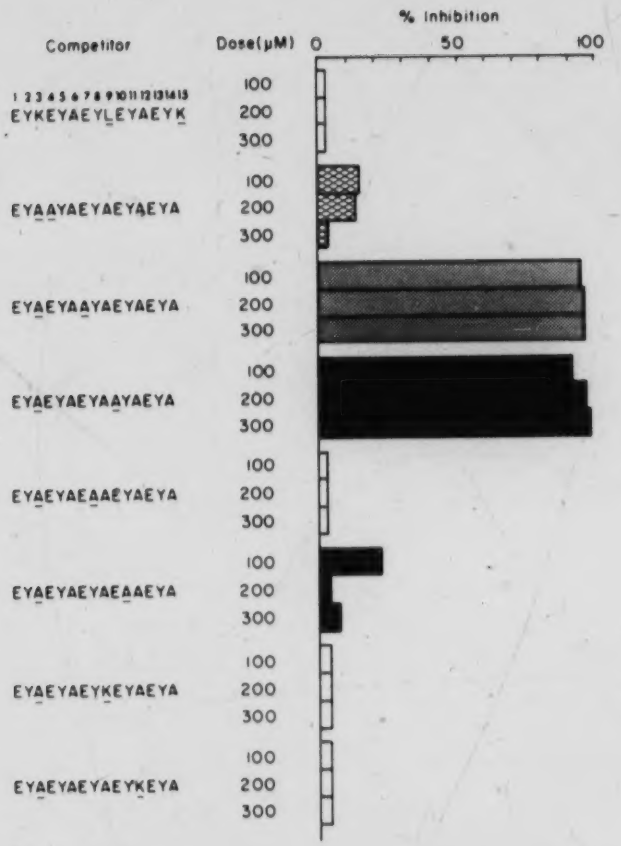

Figure 5.3. Competitive inhibition of A.1.1 response to EYK(EYA)$_4$ by peptides with multiple amino acid substitutions. T cell hybrids were incubated for 24 h with TA3 cells, stimulating antigen EYK(EYA)$_4$ at 40 μM and competing peptides at the indicated doses. Supernatants were tested for the presence of IL-2 and percent inhibition was calculated as described in Materials and Methods.

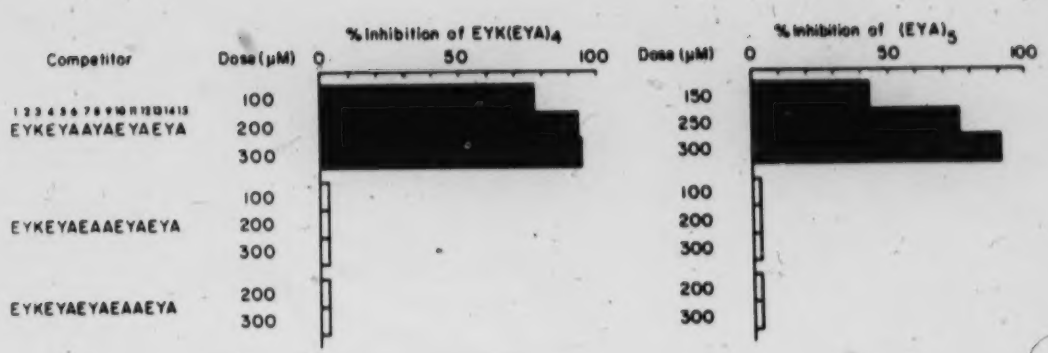

Figure 5.4. Competitive inhibition of B.1.1 response to EYK(EYA)$_4$ and (EYA)$_5$ by substituted peptides with Lys at position 3. T hybrids were incubated for 24 h with TA3 cells, stimulating antigen EYK(EYA)$_4$ or (EYA)$_5$ at 10 μM, and competing peptides at the indicated doses. Supernatants were tested for the presence of IL-2 and percent inhibition was calculated as described in Materials and Methods.

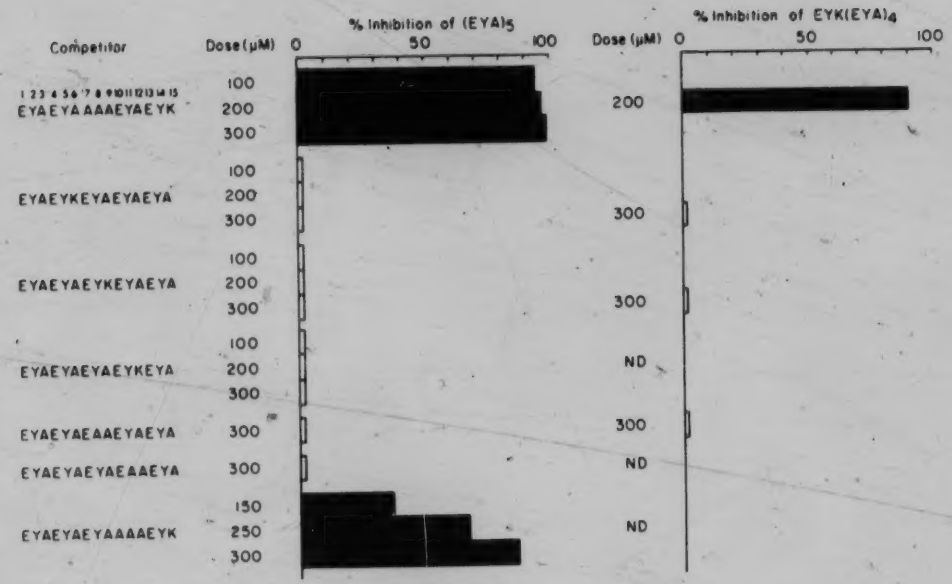

Figure 5.5. Competitive inhibition of B.1.1 response to EYK(EYA)$_4$ and (EYA)$_5$ by substituted peptides with Ala at position 3. T hybrids were incubated for 24 h with TA3 cells, stimulating antigen (EYA)$_5$ at 10 μM or EYK(EYA)$_4$ at 40 μM, and competing peptides at the indicated doses. Supernatants were tested for the presence of IL-2 and percent inhibition was calculated as described in Materials and Methods. ND: not determined.

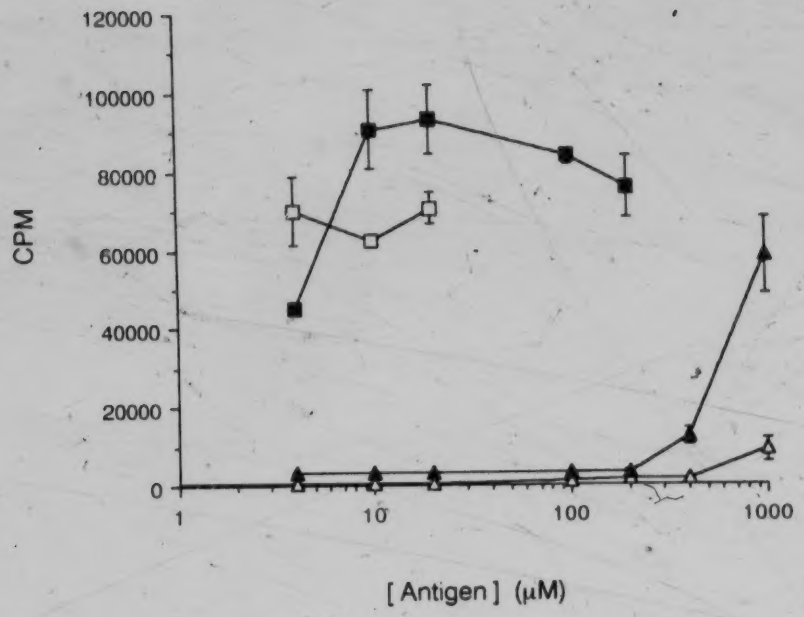

Figure 5.6. Substitution of the glutamic acid at position 7 of EYK(EYA)$_4$ by an alanine increases efficiency of peptide binding to the APC. TA3 cells were prepulsed with EYK(EYA)$_4$ (triangles) or EYKEYAAYA(EYA)$_2$ (squares) at the indicated doses for 2 h (open symbols) or 18 h (closed symbols) and washed. A.1.1 cells were added for an additional 18 h and culture supernatants were tested for the presence of IL-2 as described in Materials and Methods. Results represent the mean ± standard deviation of triplicate cultures. CPM of cultures without antigen were between 750 and 2700.

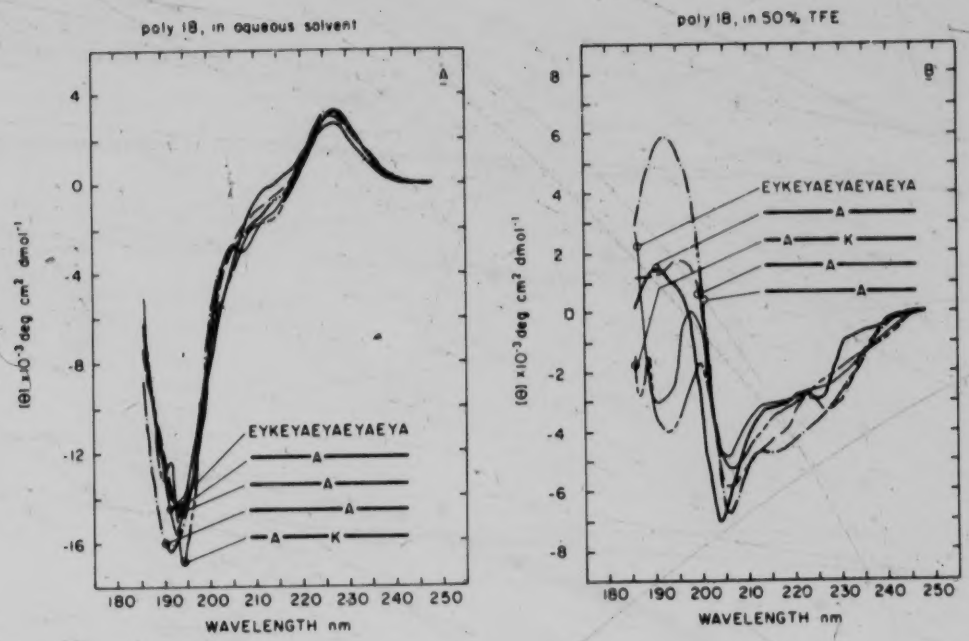

Figure 5.7. CD spectra of poly 18-related peptides in aqueous solution *(panel A)* and 50% trifluoroethanol *(panel B)*. Mean residue ellipticity [θ] was calculated as described in Materials and Methods. The α-helical content of each peptide in TFE, estimated with the Contin program [13], was: $EYK(EYA)_4$: 15%; EYKEYAEAA$(EYA)_2$, 11%; $(EYA)_2EYK(EYA)_2$, 17%; EYKEYAAYA$(EYA)_2$, 10%; EYKEYAEYAAYAEYA, 17%.

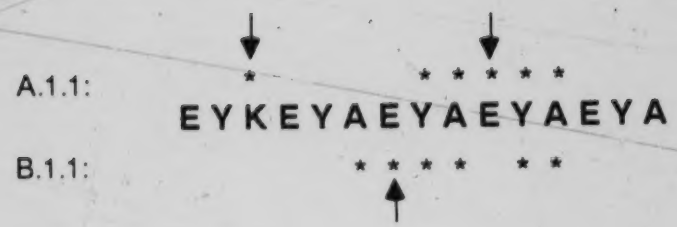

Figure 5.8. T cell receptor and Ia interaction residues of the antigenic peptide EYK(EYA)$_4$. Asterisks shown above and below the peptide sequence identify critical sites for the activation of clones A.1.1 and B.1.1, respectively. All of these residues are implicated in interactions with Ia. Top and bottom arrows indicate residues involved in interactions with the T cell receptor of A.1.1 and B.1.1, respectively.

REFERENCES

1. **Schwartz, R.H.** 1985. T lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. *Ann.Rev.Immunol. 3*:237-261.

2. **Heber-Katz, E., D. Hansburg, and R.H. Schwartz.** 1983. The Ia molecule of the antigen-presenting cell plays a critical role in immune response gene regulation of T cell activation. *J.Mol.Cell.Immunol. 1*:3-14.

3. **Buus, S., A. Sette, S.M. Colon, C. Miles, and H.M. Grey.** 1987. The relation between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunogenic peptides. *Science 235*:1353-1358.

4. **Guillet, J.-G., M.-Z. Lai, T.J. Briner, S. Buus, A. Sette, H.M. Grey, J.A. Smith, and M.L. Gefter.** 1987. Immunological self, nonself discrimination. *Science 235*:865-870.

5. **Werdelin, O.** 1982. Chemically related antigens compete for presentation by accessory cells to T cells. *J.Immunol. 129*:1883-1891.

6. **Rock, K.L., and B. Benacerraf.** 1983. Inhibition of antigen-specific T lymphocyte proliferation by structurally related Ir gene-controlled polymers. *J.Exp.Med. 157*:1618.

7. **Guillet, J.-G., M.-Z. Lai, T.J. Briner, J.A. Smith, and M.L. Gefter.** 1986. Interaction of peptide antigens and class II major histocompatibility complex antigens. *Nature 324*:260-262.

8. **Allen, P.M., G.R. Matsueda, R.J. Evans, J.B.Jr. Dunbar, G.R. Marshall, and E.R. Unanue.** 1987. Identification of the T-cell and Ia contact residues of a T-cell antigenic epitope. *Nature 327*:713-715.

9. **Sette, A., S. Buus, S. Colon, J.A. Smith, C. Miles, and H.M. Grey.** 1987. Structural characteristics of an antigen required for its interaction with Ia and recognition by T cells. *Nature 328*:395-399.

10. **Pincus, M.R., F. Gerewitz, R.H. Schwartz, and H.A. Scheraga.** 1983. Correlation between the conformation of cytochrome c peptides and their stimulatory activity in a T-lymphocyte proliferation assay. *Proc.Natl.Acad.Sci.USA 80*:3297-3300.

11. **DeLisi, C., and J.A. Berzofsky.** 1985. T-cell antigenic sites tend to be amphipathic structures. *Proc.Natl.Acad.Sci.USA 82*:7048-7052.

12. **Boyer, M., A. Fotedar, Z. Novak, E. Fraga, and B. Singh.** 1988. Identification of critical residues. (UnPub)

13. **Provencher, S.W., and J. Glockner.** 1981. Estimation of globular protein secondary structure from circular dichroism. *Biochemistry 20*:33-37.

14. **Barton, M., B. Singh, and E. Fraga.** 1977. Synthetic polypeptide antigens of defined geometry. *J.Am.Chem.Soc. 99*:8491-8498.

15. **Ramachandran, J., A. Berger, and E. Katchalski.** 1971. Synthesis and physiochemical properties in aqueous solution of the sequential polypeptide poly(Tyr-Ala-Glu). *Biopolymers 10*:1829-1851.

16. **Rothbard, J.B., R.I. Lechler, K. Howland, V. Bal, D.D. Eckels, R. Sekaly, E.O. Long, W.R. Taylor, and J.R. Lamb.** 1988. Structural model of HLA-DR1 restricted T cell antigen recognition. *Cell 52*:515-523.

17. **Rothbard, J.B., and W.R. Taylor.** 1988. A sequence pattern common to T cell epitopes. *EMBO J. 7*:93-100.

18. **Sette, A., S. Buus, S. Colon, C. Miles, and H.M. Grey.** 1988. I-Ad-binding peptides derived from unrelated protein antigens share a common structural motif. *J.Immunol. 141*:45-48.

CHAPTER 6

A critical residue of the antigen EYK(EYA)$_4$ owes its role to a neighbouring amino acid[1]

Helper T cells recognize antigen in the context of Class II histocompatibility molecules (Ia) expressed on the surface of accessory cells (reviewed in ref. [1]). Several lines of evidence support the concept of two functionally distinct sites on antigen: the epitope, involved in interaction with the T cell receptor and the agretope, interacting with Ia [2-6]. Distinct sites have been identified on several peptide antigens and ascribed to the critical amino acid residues of these antigens [1,7-9]. In these analyses, critical amino acid residues are assumed to interact either with the T cell receptor or with Ia. In most cases, however, it is not possible to formally rule out that these residues may be exerting their influence indirectly, by acting, for example, on the conformation of the antigen. In this chapter, we present clear evidence of such an indirect effect by a critical residue, whose role depends on the presence of a specific amino acid a few positions away in the antigen.

We reported earlier [10] on the characterization of two I-A^d restricted T cell hybridomas, A.1.1 and B.1.1,

[1] A version of this chapter has been submitted for publication: Boyer, M., Novak, Z., Fotedar, A., Fraga, E. and Singh, B. Science.

specific for poly 18, poly EYK(EYA)$_5$, a synthetic, alpha-helical polypeptide antigen [11-13]. The fine specificity of these hybridomas was determined using minimum size synthetic peptides of 15 amino acids, with sequences related to poly 18. With this approach, we found that A.1.1 only responds to lysine containing fragments of poly 18 while B.1.1 could be stimulated by non-lysine containing peptides. Based on the recognition patterns of these hybridomas, two major antigenic determinants of poly 18 were defined: the lysine containing EYK(EYA)$_4$ which is recognized by A.1.1 and (EYA)$_5$, seen by B.1.1. Amino acid residues at positions 3 and 9 of the 15 residue peptide EYK(EYA)$_4$ (amino acid residues were numbered 1-15 from N-terminus to C-terminus) were found to be critical for the activation of A.1.1, as lysine and alanine respectively, were required at these positions. This is shown in Table 6.1 and Figure 6.1 by the absence of A.1.1 response to (EYA)$_5$ and EYKEYAEYLEYAEYA. In order to evaluate the role of glutamic acid and tyrosine residues in the antigen, peptide analogs were synthesized containing single amino acid substitutions and these were tested for their ability to stimulate A.1.1 to release IL-2. Table 6.1 shows that replacing Tyr 8, Glu 10 and Tyr 11 of EYK(EYA)$_4$ by alanine rendered the resulting peptides inactive and thus identified these residues as critical for activation. Substitutions at other positions of EYK(EYA)$_4$ affected potency of the response in varying degrees but did not reveal a critical role for the amino acids involved.

As previously mentioned [10], $(EYA)_5$ did not stimulate A.1.1. Substituted peptides based on this sequence were also found to be non-stimulatory (Table 6.1) . Surprisingly, however, $EYAEAA(EYA)_3$ could elicit a response from A.1.1, in spite of the absence of the critical lysine residue at position 3. As shown in Figure 6.1, this peptide was somewhat less potent than $EYK(EYA)_4$, but was able to stimulate both on normal and glutaraldehyde-treated antigen presenting cells (APC). The ability of fixed TA3 to function as APC indicated that antigen processing [14] was not required for activation of A.1.1, as we have shown elsewhere [15], (Chap. 3), and suggested that $EYK(EYA)_4$ and $EYAEAA(EYA)_3$ were directly recognized by the hybridoma. To rule out the possibility that $EYAEAA(EYA)_3$ was somehow mitogenic, the peptide was tested on the beef insulin specific T cell hybridoma A20.2.15 [16] and as Table 6.2 shows, was found to have no effect on this T hybrid. Moreover, A.1.1 appeared to recognize $EYAEAA(EYA)_3$ in the same way as $EYK(EYA)_4$, as suggested by competition experiments (Fig. 6.2). Substituted peptides which did not activate A.1.1 were used to compete out $EYK(EYA)_4$ and $EYAEAA(EYA)_3$ in 24 h cultures of T cell hybrids and APC. Non-stimulatory peptides which competed against $EYK(EYA)_4$ also competed against $EYAEAA(EYA)_3$, suggesting similar recognition of both antigens by A.1.1.

The data presented here show that there is a requirement for a lysine at position 3 of $EYK(EYA)_4$ to stimulate A.1.1. However an alanine residue at position 5 instead of a

tyrosine obviates this requirement. Thus, the critical role of Lys 3 is controlled by Tyr 5. This clearly demonstrates that functional interactions occur between amino acid residues within a peptide antigen, and provides evidence that the role of critical residues may extend beyond contacting the T cell receptor or Ia, to include other functions such as, for example, stabilizing the conformation of the peptide. The possibility of such occurences has been suggested [17,18], but our data provide a direct demonstration of their existence. Furthermore, we found that similar interactions were required in antigens that stimulate B.1.1. Peptides $(EYA)_2AYA(EYA)_2$ and $EYKEYAAYA(EYA)_2$ (Table 6.1) have single substitutions at position 7 of the stimulatory antigens $(EYA)_5$ and $EYK(EYA)_4$, respectively. However, only $(EYA)_2AYA(EYA)_2$ elicits a response from B.1.1 suggesting that Ala 7 is interacting with Lys 3 to render $EYKEYAAYA(EYA)_2$ non-antigenic. Interactions within antigens have been reported in the T cell response to hen egg lysozyme [19] and to cytochrome c [20,21]. However, the effects were the result of antigen processing events which occurred prior to T cell recognition. In contrast, our data provide a striking example of functional interaction between amino acids of a peptide antigen which directly affects recognition. The precise nature of the interaction seen here is not clear at the present time. The side chains of residues 3 and 5 may be involved only with each other, stabilizing the conformation of the peptide for appropriate interaction in the antigen

binding groove of the Ia molecule [22], or they may be associating with the T cell receptor and/or Ia in a complementary way, the presence of Tyr 5 in the ternary complex requiring the presence of Lys 3 for proper interaction in the binding site. Structural studies on these peptides will be important to provide answers to these questions.

TABLE 6.1

Response of clones A.1.1 and B.1.1 to substituted peptides

Antigens	IL-2 Response CPM (x 10^{-2})		
	A.1.1		B.1.1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15	Expt 1	Expt 2	
None	1	18	15
E Y K E Y A E Y A E Y A E Y A	556	1716	2500
____A_____	384	1637	2035
_____A_____	532	678	5
_____A_____	237	1673	3
_____A_____	0.7	20	2
_____A_____	1	15	104
_____A_____	2	15	3
_____L_____	1	ND	ND
A_A_____K	ND	1186	ND
_____A_A_K	ND	1056	ND
___A_____	ND	20	2295
___A_A_____	18	18	710
___A__A_____	515	638	3
___A_____A_____	ND	13	288
___A_____A_____	0.6	18	1
___A__A_____A_____	1	15	ND
___A__A_____A_____	0.9	17	ND
___A_____A_A_____K	1	19	ND
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15			

A.1.1 or B.1.1 cells (5 x 10^4) were cultured in the presence of the B cell hybridoma TA3 (1-2.5 x 10^4 cells) as APC and peptides at 200 μM (expt. 1 and B.1.1) or 100 μM (expt. 2). Supernatants from 24 h cultures were tested for their ability to stimulate [^{3}H]-thymidine uptake by CTLL. Results represent the mean of triplicate cultures. S.D. were less than 15% of the mean. ND: not determined. Peptides were synthesized and

purified as described in chapter 4. For antigen presentation, TA3, an I-$A^{d/k}$/I-$E^{d/k}$ bearing B cell hybridoma [23] was used. Culture medium was RPMI 1640 containing 10mM HEPES, 2mM glutamine, 5 x 10^{-5}M 2-mercaptoethanol, 10% heat inactivated fetal calf serum, and 1 U/mL penicillin-streptomycin (Gibco). CTLL were cultured (10^4 cells/well) for 40 h with 10% primary supernatant from T hybrid, APC and antigen cultures. The degree of stimulation was measured by the incorporation of [^{3}H]-thymidine (40 kBq/well). Amount of primary supernatant in culture was determined to stimulate in the linear portion of the CTLL dose response curve.

TABLE 6.2

Response of beef insulin specific T hybridoma A20.2.15 to EYAEAA(EYA)$_3$

Antigen	Dose	IL-2 Response (CPM ± SD)
None	--	2115±465
Beef insulin	15 μM	145422±9000
EYAEAA(EYA)$_3$	200 μM	672±125

A20.2.15, a beef insulin reactive, I-A^d-restricted T cell hybridoma was previously described [16]. Cells were cultured like A.1.1 as in Table 6.1 legend and amount of stimulation was measured using CTL-L as described. Results represent the mean ± standard deviation of triplicate cultures.

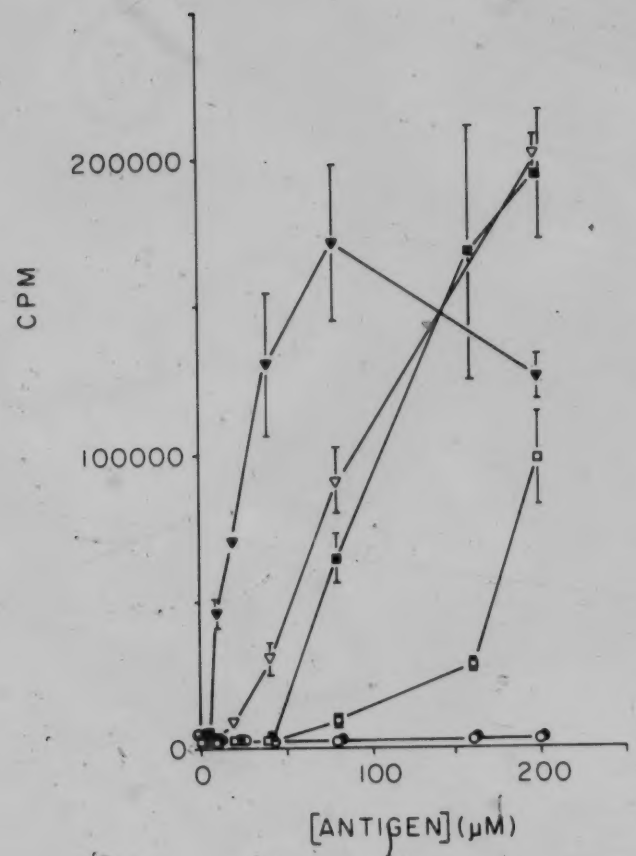

Figure 6.1. Response of T hybrid A.1.1 to EYK(EYA)$_4$ (triangles), EYAEAA(EYA)$_3$ (squares) and (EYA)$_5$ (circles) presented by normal (closed symbols) and glutaraldehyde-fixed (open symbols) TA3 cells. Each point represents the mean ± standard deviation of triplicate cultures. A.1.1 was cultured as described in Table 6.1 legend, and amount of stimulation was measured using CTL-L as described. For glutaraldehyde fixation, TA3 cells were treated as previously described (Chapter 3).

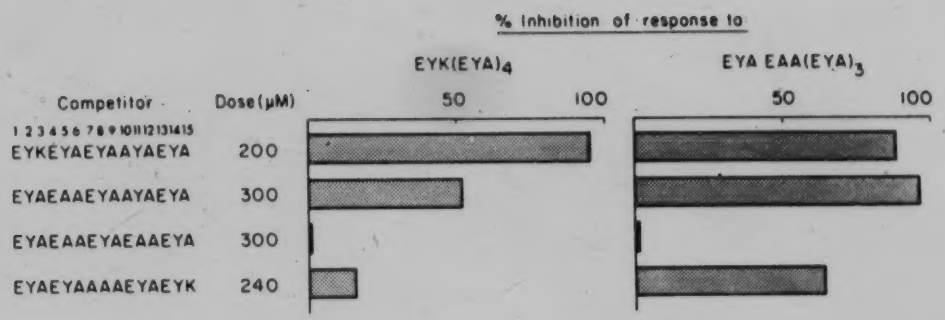

Figure 6.2. Competitive inhibition of clone A.1.1 response to $EYK(EYA)_4$ and $EYAEAA(EYA)_3$ by substituted peptides. A.1.1 was incubated for 24 h with TA3 cells, stimulating antigens $EYK(EYA)_4$ or $EYAEAA(EYA)_3$ at 40 μM or 100 μM respectively and competing peptides at the indicated doses. Supernatants were tested for the presence of IL-2 as described in Table 1. Percent inhibition was calculated as previously described (Chapter 5).

REFERENCES

1. **Schwartz, R.H.** 1985. T lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. *Ann.Rev.Immunol. 3*:237-261.

2. **Heber-Katz, E., D. Hansburg, and R.H. Schwartz.** 1983. The Ia molecule of the antigen-presenting cell plays a critical role in immune response gene regulation of T cell activation. *J.Mol.Cell.Immunol. 1*:3-14.

3. **DeLisi, C., and J.A. Berzofsky.** 1985. T-cell antigenic sites tend to be amphipathic structures. *Proc.Natl.Acad.Sci.USA 82*:7048-7052.

4. **Rothbard, J.B.** 1986. Peptides and the cellular immune response. *Ann.Inst.Pasteur 137E*:518-528.

5. **Guillet, J.-G., M.-Z. Lai, T.J. Briner, S. Buus, A. Sette, H.M. Grey, J.A. Smith, and M.L. Gefter.** 1987. Immunological self, nonself discrimination. *Science 235*:865-870.

6. **Buus, S., A. Sette, S.M. Colon, C. Miles, and H.M. Grey.** 1987. The relation between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunogenic peptides. *Science 235*:1353-1358.

7. **Allen, P.M., G.R. Matsueda, R.J. Evans, J.B.Jr. Dunbar, G.R. Marshall, and E.R. Unanue.** 1987. Identification of the T-cell and Ia contact residues of a T-cell antigenic epitope. *Nature 327*:713-715.

8. **Sette, A., S. Buus, S. Colon, J.A. Smith, C. Miles, and H.M. Grey.** 1987. Structural characteristics of an antigen required for its interaction with Ia and recognition by T cells. *Nature 328*:395-399.

9. **Fox, B.S., C. Chen, E. Fraga, C.A. French, B. Singh, and R.H. Schwartz.** 1987. Functionally distinct agretopic and epitopic sites. Analysis of the dominant T cell determinant of moth and pigeon cytochromes c with the use of synthetic peptide antigens. *J.Immunol. 139*:1578-1588.

10. **Fotedar, A., M. Boyer, W. Smart, J. Widtman, E. Fraga, and B. Singh.** 1985. Fine specificity of antigen recognition by T cell hybridoma clones specific for poly-18: a synthetic polypeptide antigen of defined sequence and conformation. *J.Immunol. 135*:3028-3033.

11. **Barton, M., B. Singh, and E. Fraga.** 1977. Synthetic polypeptide antigens of defined geometry. *J.Am.Chem.Soc. 99*:8491-8498.

12. **Singh, B., E. Fraga, and M. Barton.** 1978. Characterization and genetic control of the immune response to synthetic polypeptide antigens of defined geometry. *J.Immunol. 121*:784-789.

13. **Singh, B., K.-C. Lee, E. Fraga, A. Wilkinson, M. Wong, and M. Barton.** 1980. Minimum peptide sequences necessary for priming and triggering of humoral and cell mediated immune responses in mice: use of synthetic peptide antigens of defined structure. *J.Immunol. 124*:1336-1343.

14. **Shimonkevitz, R., J. Kappler, P. Marrack, and H. Grey.** 1983. Antigen recognition by H-2-restricted T cells. *J.Exp.Med. 158*:303-316.

15. **Boyer, M., Z. Novak, A. Fotedar, and B. Singh.** 1988. Contribution of antigen processing to the recognition of a synthetic peptide antigen by specific T cell hybridomas. *J.Mol.Rec. 1*:99-106.

16. **Morinaga, T., A. Fotedar, B. Singh, T.G. Wegmann, and T. Tamaoki.** 1985. Isolation of cDNA clones encoding a T-cell receptor beta chain from a beef insulin specific hybridoma. *Proc.Natl.Acad.Sci.USA 82*:8163-8167.

17. **Rothbard, J.B., R.I. Lechler, K. Howland, V. Bal, D.D. Eckels, R. Sekaly, E.O. Long, W.R. Taylor, and J.R. Lamb.** 1988. Structural model of HLA-DR1 restricted T cell antigen recognition. *Cell 52*:515-523.

18. **Sette, A., S. Buus, S. Colon, C. Miles, and H.M. Grey.** 1988. I-Ad-binding peptides derived from unrelated protein antigens share a common structural motif. *J.Immunol. 141*:45-48.

19. **Shastri, N., A. Miller, and E.E. Sercarz.** 1986. Amino acid residues distinct from the determinant region can profoundly affect activation of T cell clones by related antigens. *J.Immunol. 136*:371.

20. **Fox, B.S., F.R. Carbone, R.N. Germain, Y. Paterson, and R.H. Schwartz.** 1988. Processing of a minimal antigenic peptide alters its interaction with MHC molecules. *Nature 331*:538-540.

21. **Kovac, Z., and R.H. Schwartz.** 1985. The molecular basis of the requirement for antigen processing of pigeon cytochrome c prior to T cell activation. *J.Immunol. 134*:3233-3240.

22. **Brown, J.H., T. Jardetzky, M.A. Saper, B. Samraoui, P.J. Bjorkman, and D.C. Wiley.** 1988. A hypothetical model of the foreign antigen binding site of Class II histocompatibility molecules. *Nature 332*:845-850.

23. **Glimcher, L.G., T. Hamano, R. Asofsky, E. Heber-Katz, S. Hedrick, R.H. Schwartz, and W.E. Paul.** 1982. I region-restricted antigen presentation by B cell-B lymphoma hybridomas. *Nature 298*:283-284.

CHAPTER 7

General conclusions and future prospects

In this study, we examined the antigen recognition requirements of T cell hybridomas derived from BALB/cCr immune responses to the synthetic polypeptide poly 18 and related peptides. The principal findings emerging from this analysis are the following:

1. Two major I-A^d restricted clonotypes were identified from the poly 18-specific T cell population represented by the hybridomas. The first clonotype, called group A and exemplified by clone A.1.1, is specific for the lysine-containing portion of poly 18, while the second, group B, represented by B.1.1, recognizes preferentially, but not exclusively, a determinant without lysine.

2. Poly 18 and stimulatory peptide analogs are recognized without prior processing by the antigen presenting cell.

3. The minimum-size peptide capable of eliciting a reliable response from the T hybrids is 15 amino acids long. A 13 residue peptide, $A(EYA)_4$, stimulates B.1.1 but the response was found to be variable.

4. The minimum peptide EYK(EYA)$_4$ contains 6 critical residues. Four of these, Tyr 8, Ala 9, Tyr 11 and Ala 12, are required for the stimulation of both A.1.1 and B.1.1. They are thought to interact with Ia since they are necessary for both hybridomas and because peptides substituted at these positions fail to compete in functional assays. The other two critical residues are unique to each hybridoma: A.1.1 requires Lys 3 and Glu 10 while B.1.1 depends on Ala 6 and Glu 7. With the exception of Ala 6, these residues have been implicated in interactions with both the T cell receptor and Ia.

5. Peptide antigens also present the following features:

   a. most of the critical residues are clustered together in the center of the molecule.
   b. certain residues interact with other amino acids within the antigen in a way that critically affects hybridoma responses.
   c. there appears to be little ordered secondary structure in the peptide antigens used here, as determined by circular dichroism.
   d. a substantial amount of degeneracy in T cell recognition allows several substitutions to be introduced in the antigens which, in some cases,

result in analogs of increased potency. This may be useful for the generation of peptide vaccines.

The conclusions emerging from this study highlight, by their diversity, the complexity of the antigen recognition event which leads to the activation of T cells. This feature could not have been predicted at the outset when the parameters of the study were defined. Indeed, these were chosen to simplify the system as much as possible in order to maximize the information which could be derived from the data. This is exemplified by the choice of antigen: the alpha-helical poly 18 is a simple molecule with limited diversity in its amino acid composition and a well defined, ordered secondary structure. However, it engages in a complex set of interactions with Ia and the T cell receptor and amino acids within the antigen affect T cell recognition by their relationships with each other. At the present time, the mechanisms by which these multiple interactions operate are unknown. It may be that they will only be sorted out by co-crystalizing a tri-molecular complex of T cell receptor, antigen and Ia and this achievement may take some time. A promising avenue is offered by nuclear magnetic resonance (NMR) spectroscopy since this approach can be used to study molecules in solution. Preliminary NMR studies of some of our peptide antigens, not reported in this thesis, confirmed our circular dichroism data and opened the door to the possibility of resolving the conformation of these antigens

as it is induced in the non-polar solvent trifluoroethanol. These studies could provide important clues to our understanding of the structure of antigen in the environment of the T cell receptor and Ia.

One of the interesting elements of poly 18 recognition is the heteroclitic response to a number of analogs of the antigen. The increased response of A.1.1 to EYKEYAAYA$(EYA)_2$ and of B.1.1 to EYKEYAEYLEYAEYK are notable. Using these peptides as parent sequences and generating analogs with single amino acid substitutions could provide an approach to determine if critical positions are shifted with respect to those in EYK$(EYA)_4$ and if so, what rules can be uncovered which specify how critical positions are selected.

Finally, taking advantage of the degeneracy of T hybridoma responses, antigens modified with photoaffinity reagents at or near critical positions could provide very useful probes to identify the combining site of the T cell receptor or putative subsites on Ia. These reagents can be used to cross-link labelled antigen to one or both macromolecules making up the trimolecular complex, and the site where they bind can then be identified by peptide fingerprinting.

In conclusion, the poly 18 system has provided a number of valuable insights into the phenomenon of antigen

recognition by T cells and presents many interesting features which can provide the basis for future avenues of research.